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ENDOCRINOLOGY

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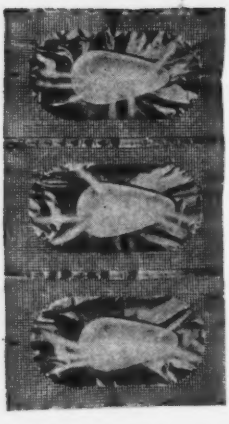

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CARL R. MOORE

1892–1955

ON OCTOBER 16, 1955 death came to Carl R. Moore at the peak of a distinguished career as a scientist and teacher. In his sixty-three years of life he had risen to a position of eminence in his chosen field of endocrinology. This eminence he attained relatively early.

As a young boy he lived on a Missouri farm, a period which he loved to recall; his early schooling was in Springfield, Missouri—his degrees of B.S. and M.A. were obtained there from Drury College in 1913 and 1914. He then came to the University of Chicago to study for a Ph.D. degree with Frank R. Lillie, and after the degree was conferred in 1916 he remained as a member of the Zoology Department and later, as its chairman until his death.

Moore's first publication—his thesis—was on fertilization and parthenogenesis in the sea urchin *Arbacia*, but at Lillie's suggestion he turned to the problem of the freemartin and the role of sex hormones in sex differentiation. The freemartin became a leitmotiv, so to speak, in all his subsequent work, and it was never far from his thoughts although much of his most significant research did not bear directly on the problem. He himself, in

retrospect, divided his studies into three periods—an early one dealing with sex gland transplantation, castration, vasoligation, cryptorchidism and scrotal function; a second in which bioassay methods for male hormone were developed and hormone effects and interrelationships were studied, and a third, when synthetic sex hormones were available, in which he approached the problem of sex differentiation by treating pregnant rats and pouch young opossums with sex hormones and by gonadectomy of young opossums.

The most important contributions among his eighty or so major publications were made in the two earlier periods—a time when the field of endocrinology was rapidly developing and there was a wealth of new and exciting facts only waiting to be discovered and new theories to be formulated. It was here that he first made his mark and established his scientific stature. His findings not only advanced materially the understanding of endocrine problems, but they influenced and stimulated extensive research in ever widening fields. He did not solve the original problem—the freemartin—but he accomplished far more. The work that he did along the way established some of the most fundamental concepts of endocrinology.

The value of his work was widely recognized and he received many honors and awards. Among them were membership in the National Academy of Sciences; the Francis Amory award of the American Academy of Arts and Sciences, 1941; the award of the American Urological Association, 1950; the medal and certificate of award of the Endocrine Society, 1955; an honorary D.Sc. degree from Drury College.

He found time in his busy life to serve on the editorial boards of several journals and on committees—especially the Committee for Research in Problems of Sex, of the National Research Council. He was vice-president of Section F of the AAAS in 1943 and president of the Endocrine Society, 1944–46. He gave generously of his time and energy to the many graduate students who came to Chicago to work with him.

It is impossible to separate in his personality Carl Moore the man from Carl Moore the scientist. His scientific interests were his life. Enthusiastic, dynamic and vigorous until his long illness sapped his strength and robbed him of initiative, he put almost all of himself into his research, his teaching and his department. But if he was a devoted and ambitious scientist he was not a cold and remote one. His personality radiated warmth, kindliness and personal interest for others. He was a man of firm convictions which he stoutly defended but he was not bitter nor petty in his attitude toward those who disagreed with him. As a teacher he was enthusiastic and inspiring; as an administrator he was fair and just; as a friend he was warm and loyal.

He has left a permanent contribution to endocrinology and a rich memory of a vital and courageous personality to his colleagues and his other friends.

DOROTHY PRICE

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THE EFFECT OF THYROID STIMULATING HORMONE UPON THE IODIDE COLLECTING MECHANISM OF THYROID TISSUE SLICES[1]

JOHN L. BAKKE, AND NANCY LAWRENCE

(*With the technical assistance of* ANNE NEWCOMB *and* CURT WIBERG)

Veterans Administration Hospital and the Department of Medicine, University of Washington, Seattle, Washington

IN SPITE of an increasing number of techniques for the assay of thyroid stimulating hormone (TSH), none of the methods has been generally adopted. The reported assays continue to be limited in usefulness because of their complexity, variability, and limited sensitivity. Sensitivity is most important since it is desirable to measure directly and without an intervening concentration procedure the small amounts of the hormone present in body fluids. The present communication describes conditions for studying radioiodide uptake and release from beef thyroid slices as these processes were altered by the *in vitro* addition of TSH. It is hoped that these experiments will provide a basis for the development of an assay for TSH.

Tissue slice experiments have several advantages over *in vivo* systems for detecting TSH: (1) the added TSH can be confined in a small volume with the target tissue, thus avoiding dilution throughout the body as occurs in *in vivo* experiments; (2) only the isolated target tissue is present, avoiding catabolism of TSH in other tissues which may normally account for the breakdown of most of the circulating TSH (D'Angelo, 1955); (3) a large number (up to 65 in this study) of comparable slices can be prepared rapidly from a single lobe of beef thyroid and incubated together in the same incubator permitting the convenient accumulation of sufficient replicate data to validate small differences between control and experimental groups.

Surviving thyroid slices preserve many aspects of iodine metabolism found *in vivo* including the collection and concentration of inorganic iodide (Morton and Chaikoff, 1943) followed by the organic binding of this ion

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[1] Aided in part by the U. S. Public Health Service Grant No. 701 and the Washington State Initiative 171 Fund.

(Chaikoff and Taurog, 1949). Using these experiments as models, the effects of added TSH were measured on tissue oxygen consumption and on the three steps in iodine metabolism pertinent to increased thyroid hormone output; i.e., (1) the collection or trapping of inorganic iodide, (2) the synthesis of this iodide into organically bound iodine compounds and (3) the release of these compounds into the fluid environment.

Preliminary experiments in our laboratory (Bakke and Lawrence, 1954, a,b,c,d) demonstrated that 30 minutes after adding TSH to beef thyroid slices, there was an increase in oxygen consumption,[2] radioiodide uptake and organic binding but an increased release of labelled organic compounds was not observed. The stimulation of organic binding, like the stimulation of oxygen consumption, required the presence of relatively large amounts of TSH (30 U.S.P. m.u./ml. of medium[3]) in contrast to the iodide collecting mechanism which responded to as little as 1×10^{-3} m.u. of TSH/ml. of medium.

For this reason the experiments to be reported here were restricted to a study of the thyroid iodide collecting mechanism or trap, as it was altered by various conditions of incubation appropriate to measuring the effects of TSH added *in vitro*.

MATERIALS AND METHODS

Tissue

Fresh, intact beef thyroids were obtained from a nearby abattoir.[4] The glands were removed 10 to 20 minutes after the animal's death, wrapped in parafilm and packed in ice during the 15 minute transit to the laboratory. The glands were dissected free of the surrounding fat and connective tissue capsule and cut into blocks appropriate for slicing. Slices (*ca.* 75 mg.) were made with a manual Stadie-Riggs type microtome. Slices 0.45 mm. thick had a maximal QO_2 so this thickness was adopted for all experiments. All tissue handling and slicing was carried out through arm ports inside a stainless steel, glass top, refrigerated tissue box kept at 4° C and 95–100% relative humidity with a spinning disc humidifier. The slices were selected for their freedom from connective tissue and accumulated on a palette of filter paper, moistened with medium (described below), rinsed collectively three times in 200 ml. of medium for a total of 10 minutes to remove blood and easily detached tissue fragments, returned to a fresh filter paper palette and allowed to equilibrate at 4° C for 15 minutes to stabilize the increase in weight due to hydration. The individual slices were then rapidly weighed on a torsion balance and returned to numbered positions on the filter paper palette in the cold-moist box where they remained while medium was pipetted into the incubation beakers. Although these preliminaries took from one to two hours, it was determined that the slices could be stored for this period without any decrease in their iodide collecting capacity (see below).

[2] Within 30 minutes after adding 3×10^{-2} U.S.P. units of TSH, the QO_2 increased from 100 ± 9.1 μl. of tissue desoxyribonucleic acid/hour to 129.5 ± 14.7 μl./mg. ($t_{12}=8.0$; $P<0.001$). This control QO_2 is equivalent to 0.42 μl. O_2/hr./mg. of tissue wet weight, which is closely similar to the values obtained by others (Turner and Turner (1945) and Freinkel and Ingbar (1955, a)).

[3] One milliunit, hereafter abbreviated m.u., is one-thousandth of a U.S.P. unit.

[4] We are grateful for the generous cooperation of Mr. Hugh J. Brom, Plant Superintendent, Seattle Packing Company.

Medium

All slices were rinsed and incubated in freshly prepared Krebs-Henseleit Ringer's bicarbonate medium (Field, 1948) buffered to a pH of 7.4 by equilibration with 95% O_2–5% CO_2. Propylthiouracil (PTU) at a final concentration of 10^{-3} M. was present in all the medium to prevent organic binding of iodide (Wollman and Scow, 1954). The medium also contained potassium penicillin-G (10^{-3} M.) and streptomycin (10 mg.%). These antibiotics permitted 30 hours incubation without significant bacterial growth as tested by smear and culture. The effect of these antibiotics on radioiodide uptake is discussed below.

After studying the effects of varying the medium carrier iodide concentration over a wide range (see below) a level of 10 μg.% was adopted for routine use. Although it is reported (Freinkel and Ingbar (1955), Morton Chaikoff and Rosenfeld (1944)) that reagent contamination with iodide results in a minimum medium iodide concentration of 10 μg.%, we were unable to confirm this by direct chemical measurements (Barker's alkaline incineration method). Using this method which is sensitive to 0.5 μg., no detectable iodide was found in our carrier free medium, and our usual medium prepared to contain 10 μg.% carrier iodide as KI was measured to have 9.6 ± 0.5 μg.%.

For reasons to be discussed subsequently, each slice was always incubated in a volume of medium proportional to the weight of the slice.

Radioiodide tracer was present in the medium in a concentration of less than 0.1 μc./ml. which does not cause radiation damage of the tissue (Freinkel and Ingbar, 1955).

TSH powder[5] was dissolved freshly for each experiment.

Methods

The slices were incubated at 37° C in a Dubnoff metabolic shaking incubator, constructed to hold 65 beakers, under an atmosphere of 95% O_2–5% CO_2. The shaking rate was 100–120 strokes per minute.

The experiments were of two types: The first, called "*uptake experiments*," consisted of incubating the slice in radioiodide medium. At the end of incubation the radioactivity collected by the slice was counted and the per cent uptake was calculated.

The second type of experiment, called "*release experiments*," consisted of an initial uptake or loading period during which the slice concentrated radioiodide from the surrounding medium, followed by transfer of the slice into non-radioactive medium for a second period of incubation during which radioiodide was released from the slice into the medium. During transfer the slice was dipped for approximately one second in each of a series of three separate 200 ml. wash baths of non-radioactive medium to remove the radioactive medium adherent to the surface of the tissue.

At the end of incubation the slices were removed by forceps, rinsed for approximately two seconds in 200 ml. of non-radioactive medium, drained on the beaker lip for approximately one second and placed in the bottom of a counting vial. All sequential operations were timed to retain approximately the same delay for each individual slice from the start to the end of the experiment. One ml. of the incubation medium in each beaker was subsequently transferred to a counting vial.

All counting was done in a well-type scintillation detector which has a constant efficiency for volumes less than 2.5 ml. At least 6400 counts were obtained from each sample to reduce the probable counting error to less than one per cent.

The radioiodide uptake by each individual slice was determined by subtracting the final medium radioactivity from the starting value (obtained by counting an aliquot of

[5] Generously supplied by Dr. Sanford L. Steelman, Armour Laboratories, Armour #317–147; one μg. equivalent to 0.8 U.S.P. m.u.

the initial medium). In release experiments the appearance of radioactivity in the medium was measured. The radioactivity remaining in the slice at the end of the experiment was measured and knowing the initial value the per cent release was calculated. The final recovery of radioactivity varied from 97% to 103% of the starting total and the standard deviation of the mean final recovery varied between ± 0.8 and $\pm 4.0\%$ in these experiments.

Ordinarily there appeared to be no advantage in converting per cent values into the equivalent absolute iodide based upon the known carrier iodide level, nor was there any apparent advantage in presenting the data as T/M concentration ratios.[6] Because of the hyperbolic relationship between the per cent uptake or per cent release and the T/M ratio, small changes in one parameter are reflected in very large changes in the other parameter except in the middle portion of the curve. This relationship has been presented by Freinkel and Ingbar (1955). In Figure 1 of their paper, one may see how very small variations in measuring the per cent uptake may cause disproportionately large changes in the calculated T/M ratio when the per cent uptake is relatively high.

RESULTS

I. *Factors affecting iodide uptake and release*

1) *Effect of Temperature:* Radioiodide uptake increased with temperature until an optimum was reached at about 40° C, the values thereafter declining (Fig. 1). Accordingly, a temperature of 37° C was selected for all

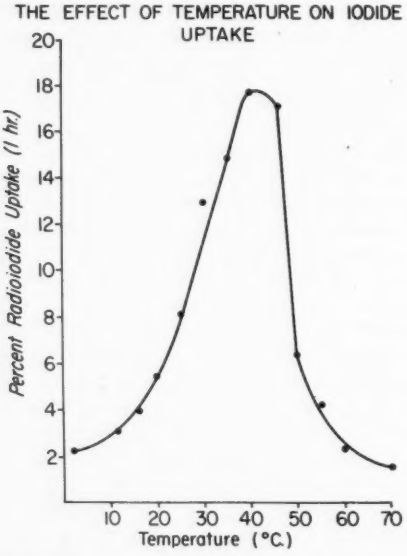

FIG. 1. Per cent radioiodide uptake by thyroid slices incubated at various temperatures; a total of 160 slices from several glands incubated individually for 1 hour in 4 ml. of medium/100 mg. of tissue. Between 2° C and 40° C the temperature coefficient is 2.0 and the net activation energy calculated with the Arrhenius equation is 13,000 calories per mole.

[6] The T/M concentration ratio may be defined as the the counts of radioiodide per gram of tissue divided by the counts per ml. of medium. It is a convenient measure of the iodide concentrating capacity when a steady state is achieved.

subsequent experiments.

2) *Effect of Aging:* Freinkel and Ingbar (1955) showed that 24 hours storage at 4° C reduced the iodide concentrating capacity of sheep thyroid slices to less than half the control (fresh) values. To evaluate the effect of shorter periods of storage which might be encountered in setting up one of our experiments, 20 slices were stored for three hours at 4° C. When incubated, these aged slices concentrated $39.2 \pm 4.1\%$ (mean $\pm$ standard deviation) of the radioiodide in 4 ml. of medium/100 mg. of tissue in one hour. Twenty fresh control slices collected $41.9 \pm 6.4\%$. The difference between these two values is not significant.

Because of the prolonged incubation used in some experiments the viability of slices was studied after 22 hours incubation at 37° C in the shaking incubator. The control group of ten slices was incubated as soon as possible after preparation. In two hours these slices collected 25.0 ± 4.53 per cent of the radioiodide offered in 10 ml./100 mg. of tissue. The ten slices aged for 22 hours collected 25.1 ± 4.92 per cent of the radioiodide. Not only was the uptake the same in the aged slices as in the fresh slices, but in both cases the iodide was completely (99.0%) flushed by 10^{-3} M. thiocyanate and was completely (98.3%) soluble in 10% trichloracetic acid, indicating that it had not been organically bound.

3) *Effect of Medium Volume:* When comparable slices were incubated one hour in various aliquots of the same radioiodide medium (all containing 10 μg.% carrier iodide and the same specific activity) the per cent radioiodide concentrated by the slice decreased as the volume of medium was increased. When these data were plotted in terms of the calculated equivalent amount of carrier iodide uptake, a plateau was reached at 0.84 μg. iodide uptake/Gm. of tissue/hour. Using this limiting value the per cent uptake for a variety of medium volumes was calculated. When these results were plotted they formed a rectangular hyperbola which appears as a straight broken line in the logarithmic plot of Figure 2. This line appeared to fit the experimental data satisfactorily except at the smallest volumes when the slice depleted the total available iodide within the one hour incubation period. If the concentration of iodide remains constant, the relationship may be expressed by a typical "clearance" equation: $PV = C$, where P is the per cent radioiodide uptake per 100 mg. of tissue per hour, V is the medium volume per 100 mg. of tissue and C is a constant. When slices of differing weights are incubated under standard conditions in identical volumes of medium, the per cent uptake will also vary, and one cannot correct these variations by dividing the uptake by the individual slice weight, as is so often done in slice-uptake experiments. For this reason the volume was always adjusted proportionally to the slice weight in each individual beaker as described above under "Method." For convenience, and in anticipation of using serum, the volume of the medium was ordinarily limited to 4 ml./100 mg. of tissue.

4) *Effect of Medium Iodide Concentration:* VanderLaan and VanderLaan

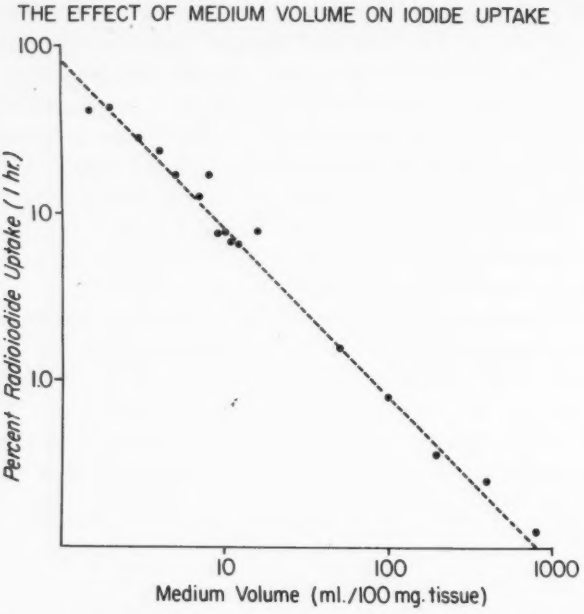

FIG. 2. Per cent radioiodide uptake from various volumes of incubation medium; a total of 200 slices from several glands incubated individually for 1 hour in radioiodide medium; initially containing 10 μg.% carrier iodide. The dotted line is calculated on the assumption that PV = C; i.e., per cent uptake $\times$ medium volume/100 mg. of tissue = 0.84 μg. iodide uptake/Gm. of tissue/hr. (see text).

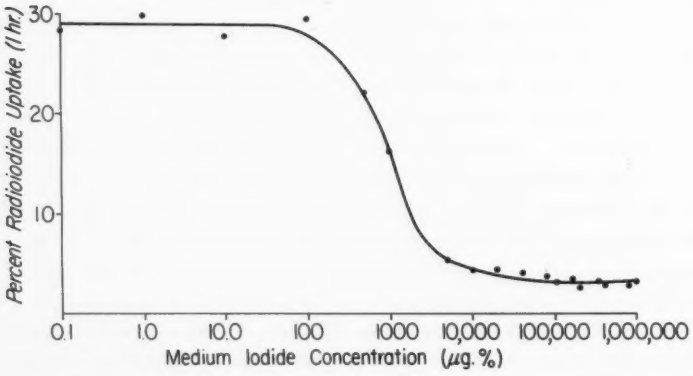

FIG. 3. Per cent radioiodide uptake from medium containing various concentrations of carrier iodide; a total of 162 slices from several glands incubated individually for 1 hr. in 4 ml. of medium/100 mg. of tissue.

(1947) have shown that the thyroid iodide collecting mechanism operates independently of the serum iodide concentration over a wide range and uptake was decreased only when the serum iodide level was relatively high. Figure 3 confirms this observation *in vitro*. As can be seen, the per cent radioiodide uptake remained constant as the medium carrier iodide concentration was increased from trace levels to 100 μg.%. When the medium iodide concentration was above 100 μg.% the one hour radioiodide uptake declined sharply until it finally leveled off near 2% when the iodide concentration exceeded 10 mg.%. With a constant volume in the equation $PV = C$, the per cent uptake is also a constant until saturation of the iodide concentrating mechanism alters the relationship.

5) *Effect of Incubation Period:* The period of incubation was varied from 10 minutes to 30 hours. Because prolonged incubation in a small volume of medium allowed almost complete uptake of the available iodide, experiments were also performed in the relatively infinite volume of 800 ml. per 100 mg. of tissue.[7] Figure 4 shows the results of several of these experiments. Figure 4a shows that at least 10 hours were necessary for the tissue radioactivity to reach a maximum value, after which time it declined. This decline may represent loss of tissue viability although experiments with aged tissue described above do not support this interpretation. In Figure 4b release experiments do not show a similar decline because the slice has already released approximately 100% of its radioactivity after 16 hours. Figure 4c shows that uptake in small volumes depleted the medium radioiodide in contrast to release experiments in a small volume (Fig. 4d) which appeared to achieve a steady state after about 6 hours. For these reasons uptake experiments in small volumes were not prolonged beyond 3 or 4 hours without presenting the slice with a fresh supply of medium iodide, in contrast to release experiments which were prolonged as long as 24 hours without changing the medium.

6) *Effect of Antibiotics:* In performing experiments of more than a few hours duration the addition of 10^{-3}M. penicillin and 10 mg.% streptomycin in the medium was necessary to prevent heavy bacterial growth. Although antibiotics were probably unnecessary in shorter incubation periods they were used throughout for uniformity even though they caused a small inhibition of the one hour radioiodide uptake.

In one experiment the one hour radioiodide uptake by 25 slices incubated without antibiotics was 28.8 ± 3.1%. Twenty-five comparable slices incubated with the antibiotics concentrated 24.8 ± 5.4% of the radioiodide. This depression of 15% was of borderline significance ($P = 0.02$) and was less marked than the depression reported by Slingerland (1955),

[7] In order to accommodate volumes up to 800 ml., liter flasks were submerged in a large constant temperature water bath at 37° C, and 95% O_2–5% CO_2 was bubbled from the bottom of each flask through a scintered glass rod to ensure both mixing and gassing of the medium.

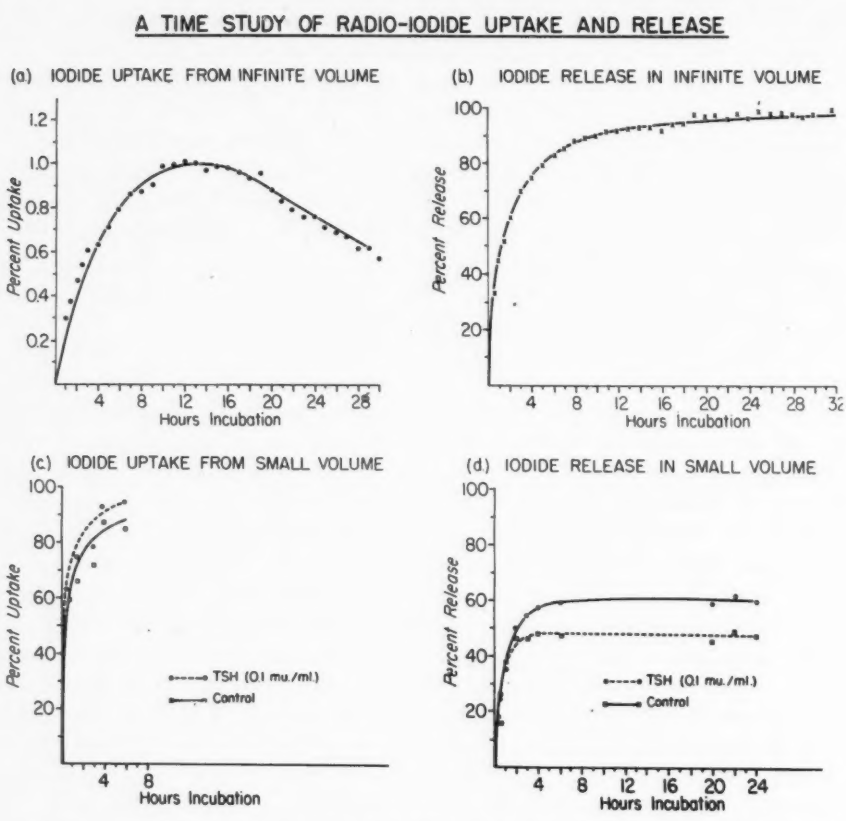

FIG. 4. A time study of per cent radioiodide uptake and release from infinite (800 ml.) and small volumes of medium.

FIG. 4a. Radioiodide uptake measured by repeatedly removing and counting the same slice as each was incubated in 800 ml. of medium/100 mg. of tissue. Data from 140 experimental observations made upon 6 slices.

FIG. 4b. Radioiodide release from 100 mg. radioactive slices into 800 ml. of medium measured by counting serial 1.0 ml. samples of the initially non-radioactive medium. Each 100 mg. slice was previously loaded with radioiodide by a 1 hr. incubation in 4 ml. of radioactive medium. Data from 384 experimental observations on 12 slices incubated individually.

FIG. 4c. Radioiodide uptake from a small volume (2 ml./100 mg. of tissue) of medium. Data from 116 slices incubated individually. After 2 hrs., TSH (8×10^{-2} m.u./ml.) had significantly enhanced the uptake of radioiodide as compared with the controls ($P < 0.001$).

FIG. 4d. Radioiodide release into a small volume (10 ml. of non-radioactive medium /100 mg. of tissue). The slices were previously loaded with radioiodide by a 1 hr. incubation in 4 ml. of radioactive medium/100 mg. of tissue. Data based upon 60 slices incubated individually. TSH (1×10^{-1} m.u./ml.) significantly enhanced the retention of radioiodide after 6 hours of incubation ($t_{23} = 5.52$; $P < 0.001$).

possibly due to difference in the tissue source and the technique used. Of more importance, further experiments showed that these antibiotics did not interfere with the response to TSH.

II. *The Effect of TSH—General Considerations*

Since the optimal conditions for iodide collection are not necessarily the same as those for TSH responsiveness, the effects of TSH were also measured in conjunction with the experimental conditions discussed above.

1) *Effect of Medium Volume:* When radioiodide uptake was measured in small volumes the depletion of medium iodide was so great that the opportunity to measure any additional uptake due to TSH as seen in Figure 4c was limited to a critically brief and variable period which led to the early abandonment of this type of experiment. When slices were transferred at hourly or two hour intervals into an aliquot of the original medium to give the slice a fresh source of labeled iodide this limitation was circumvented. Figure 5 shows a typical experiment. The per cent uptake for each incubation period was converted into the equivalent amount of inorganic iodide (calculated from the known carrier level of 10 μg.%) so the values could be added and cumulated. Although straight lines connect

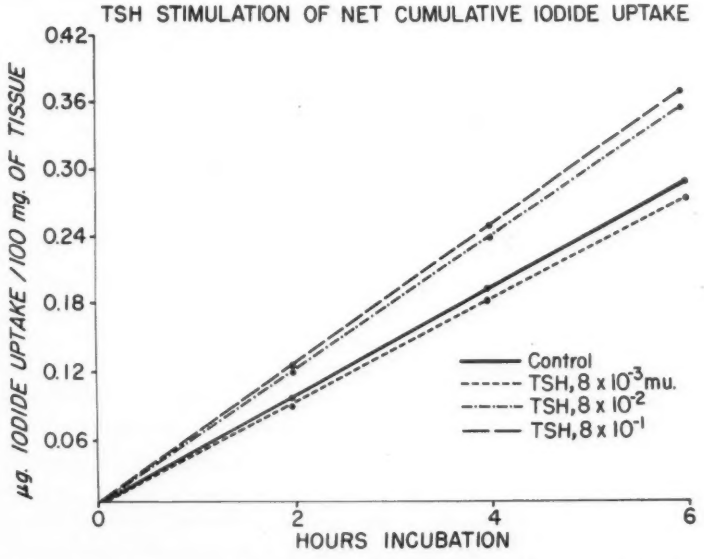

FIG. 5. A typical experiment showing TSH stimulation of the cumulative iodide uptake by the isolated iodide trap. The radioiodide medium was changed every 2 hours; 2 ml./100 mg. of tissue; 10 μg.% carrier iodide; 10 slices incubated individually per point. TSH, 8×10^{-1} and 8×10^{-2} m.u./ml. of medium significantly ($P < 0.001$) elevated the radioiodide uptake. (The straight lines are not intended to indicate a linear process; see text.)

the cumulative values, these lines are not meant to indicate that the uptake during any two hour segment was linear since the absolute (not per cent) iodide uptake is known to decline with depletion of the medium iodide. The calculated cumulative iodide uptake was increased to 130% ($P=0.01$) of the control value two hours after incubation in 8×10^{-2} m.u. TSH/ml.; an increase to 8×10^{-1} m.u. of TSH/ml. did not further increase this response and 8×10^{-3} m.u./ml. failed to increase the uptake over the control level. In further experiments of this type, 122 slices were individually incubated in a series of 732 beakers. Five concentrations of TSH ranging between the above extremes were compared with controls. At all TSH concentrations of 2×10^{-2} m.u./ml. or above, a statistically significant ($P<0.001$) response was obtained. No useful dose-response proportionality was observed.

TABLE 1. A COMPARISON OF UPTAKE *vs.* RELEASE EXPERIMENTS

	Per cent uptake[1] (from 3 ml.)	Statistical significance[2]		Per cent release[3]	Statistical significance	
TSH[4]		t_{19}	P		t_{19}	P
0	69.1 ± 6.25	—	—	44.0 ± 8.31	—	—
3×10^{-1}	74.1 ± 4.97	1.98	n.s.	28.1 ± 8.33	4.27	<0.001
	Per cent uptake[5] (from 30 ml.)			Per cent release[3]		
TSH[4]		t_{15}	P		t_{15}	P
0	7.44 ± 1.61	—	—	48.9 ± 6.14	—	—
4×10^{-2}	9.78 ± 2.13	2.48	<.04	38.2 ± 2.78	4.49	<0.001
4×10^{-1}	11.0 ± 3.39	2.68	<.04	40.4 ± 2.70	3.58	<0.001

[1] Radioiodide uptake after 5 hours incubation; 3 ml. of medium/100 mg. of tissue, all tissue from same gland; 10 beakers per datum. Mean ± standard deviation.

[2] Statistical significance calculated according to "t" test tables. A "P" value <0.02 is considered significant and >0.05 is n.s. (not significant).

[3] Radioiodide release; 1 hr. uptake and 4 hr. release, both in 4 ml. of medium/100 mg. of tissue. Mean ± standard deviation.

[4] TSH in m.u./ml. of medium.

[5] Radioiodide uptake after 5 hours incubation; 30 ml. of medium/100 mg. of tissue; all tissue from same gland; 8 beakers per datum. Mean ± standard deviation.

Variations in both uptake release medium volumes were then studied in release experiments. The volume of medium used was not a critical variable in obtaining a response to TSH. Small volumes (4–10 ml./100 mg. of tissue) were selected as being appropriate to the goal of measuring small amounts of TSH.

2) *A Direct Comparison of Uptake and Release Experiments:* Table 1 illustrates the effect of TSH on uptake and release experiments performed together in the same incubator with comparable slices from the same gland. The advantage of the release experiment over the uptake experiment was apparent even when the uptake experiment utilized a volume large enough (30 ml.) to circumvent significant depletion of the medium iodide.

3) *Effect of Medium Iodide Concentration:* Table 2(a) shows a release experiment in which one group of slices released radioiodide into medium containing 100 μg.% carrier iodide and another group released into 1000 μg.% iodide. There was a statistically significant ($P < 0.001$) TSH effect at the lower iodide level which was absent at the higher iodide level. As long as the medium iodide concentration remained below 100 μg.%, even large (100 fold) differences between the iodide concentration in the uptake and release medium had no effect on the TSH response as shown in Table 2(b).

TABLE 2. THE EFFECT OF CARRIER IODIDE CONCENTRATION ON RELEASE EXPERIMENTS

Experiment (a)[1]

TSH[2]	Per cent release (L-10; R-100)[3]	Statistical significance		Per cent release (L-10; R-1000)[4]	Statistical significance	
		t_9	P		t_9	P
0	50.0 ± 10.2	—	—	59.9 ± 5.21	—	—
8×10^{-4}	44.4 ± 8.80	1.37	n.s.	63.7 ± 7.52	—	n.s.
8×10^{-3}	40.5 ± 5.41	4.01	<0.01	60.8 ± 0.89	—	n.s.
8×10^{-2}	30.3 ± 3.47	5.63	<0.001	64.3 ± 5.86	—	n.s.
8×10^{-1}	30.8 ± 7.60	5.63	<0.001	53.3 ± 5.52	1.16	n.s.

Experiment (b)[5]

TSH[2]	Per cent release (L-0.1; R-10)	Statistical significance		Per cent release (L-10; R-20)	Statistical significance	
		t_{11}	P		t_{11}	P
0	80.7 ± 7.42	—	—	83.5 ± 4.24	—	—
2×10^{-2}	51.8 ± 7.94	11.9	<0.001	50.4 ± 10.5	13.0	<0.001

[1] All tissue from the same gland; incubated 1 hour in radioiodide medium; 10 μg.% carrier iodide; 4 ml./100 mg. of tissue; then released in non-radioactive medium with the iodide concentration shown; 10 ml./100 mg. of tissue. The average 1 hour uptake was 45.0% in both groups, 5 beakers per datum.

[2] TSH in m.u./ml. of medium.

[3] (L-10; R-100) indicates that the slices were loaded in medium containing 10 μg.% carrier iodide and released in 100 μg.% carrier iodide.

[4] (L-10; R-1000) indicates that the slices were loaded in medium containing 10 μg.% carrier iodide and released in 1000 μg.% carrier iodide.

[5] All tissue from the same gland; details as above, except carrier iodide concentrations which are different as indicated in brackets. The average 1 hour uptake was 30.4% in both groups; 6 beakers per datum. Mean ± standard deviation.

Subsequent experiments with TSH were always performed in medium with 10 μg.% carrier iodide.

4) *Time of Adding TSH:* In release experiments TSH could be added to either the uptake period, the release period or both of the incubation periods. A study was made to determine the optimal choice. In several experiments (comprising a total of 160 slices) it was demonstrated that it did not make a statistically significant difference whether the TSH was present in either the uptake period or the release period, or both. This may indicate

that in those experiments in which TSH was present only during the one hour uptake period it either became bound to the tissue or initiated changes in the tissue which persisted into the release period. Experiments to determine the minimum necessary period of exposure to TSH, and the rate at which thyroid slices bind or inactivate TSH, will be reported separately. In subsequent experiments described in this report, TSH was present in both periods of incubation.

5) *Effect of Incubation Period:* Table 3 illustrates one of many experiments designed to reveal the optimal incubation periods for radioiodide uptake and release in release experiments with TSH. It appeared that the duration of the uptake period was critical. As mentioned above, this was true whether TSH was present or absent in the uptake period. The effect

TABLE 3. THE EFFECT OF INCUBATION TIME ON RELEASE EXPERIMENTS[1]

Loading period (hrs.)	Per cent radioiodide uptake	Releasing period (hrs.)	Per cent radioiodine release		Statistical Significance	
			Control	TSH[2]	t_{15}	P
½	20.3 ± 3.03	3	51.0 ± 5.98	47.0 ± 3.14	1.68	n.s.
		4	51.3 ± 6.20	46.3 ± 7.64	1.75	n.s.
		5	49.5 ± 6.24	43.1 ± 7.62	2.37	<0.05
1	30.1 ± 3.60	3	53.3 ± 3.74	44.9 ± 4.26	4.19	<0.001
		4	55.4 ± 3.62	44.3 ± 4.68	5.31	<0.001
		5	51.7 ± 5.98	41.7 ± 3.80	4.00	<0.001
1½	37.7 ± 4.40	3	48.6 ± 4.68	46.2 ± 6.30	1.08	n.s.
		4	50.8 ± 6.50	47.0 ± 7.91	1.36	n.s.
		5	47.3 ± 6.21	43.1 ± 7.53	1.58	n.s.

[1] All tissue from same gland; eight beakers per datum; 4 ml. of medium/100 mg. of tissue; 10 μg.% carrier iodide. Data are mean ± standard deviation.
[2] TSH, 8×10^{-2} m.u./ml. of medium.

of TSH was maximal when the uptake period lasted approximately one hour and the releasing incubation was four hours or longer. The releasing incubation could be prolonged to 21 hours without altering the effects of TSH. These values were highly significant statistically ($P < 0.001$). The technical inconvenience of these long-term incubations with their accompanying possibility of error due to tissue disintegration, evaporation of medium or mechanical breakdown led to the adoption of a four hour release incubation period for subsequent experiments.

6) *Specificity of the Effects of TSH:* Both uptake and release experiments were used to study the effects of other pituitary proteins including ACTH, FSH, and a special fraction of lyophilized anterior pituitary powder known to be free of TSH.[8] Each substance was tested with 10 replicate slices using

[8] Anterior pituitary powder, T-88, containing no detectable TSH when 5 mg. was injected into hypophysectomized rats; generously supplied by Dr. I. Gordon Fels, Inst. of Exp. Biol., University of California, Berkeley, California; TSH, Armour 317–147; FSH, Parke, Davis 5766; ACTH, Armour-K 54707.

a concentration of 1.0 μg./ml. of medium and compared with the same amount of TSH and with controls. Only TSH produced a statistically significant increase ($P < 0.02$) in radioiodide uptake (in uptake experiments) and an increased radioiodide retention in release experiments ($P < 0.01$). Although FSH had a small effect (statistically borderline; $P = 0.05$) in both the uptake and release experiments this could reasonably have been due to the known contamination of the FSH with TSH.

III. *Quantitative Effects of TSH on Radioiodide Release*

Based upon these findings, a preliminary series of assay release experiments (comprising a total of 146 slices) were performed, using a one hour

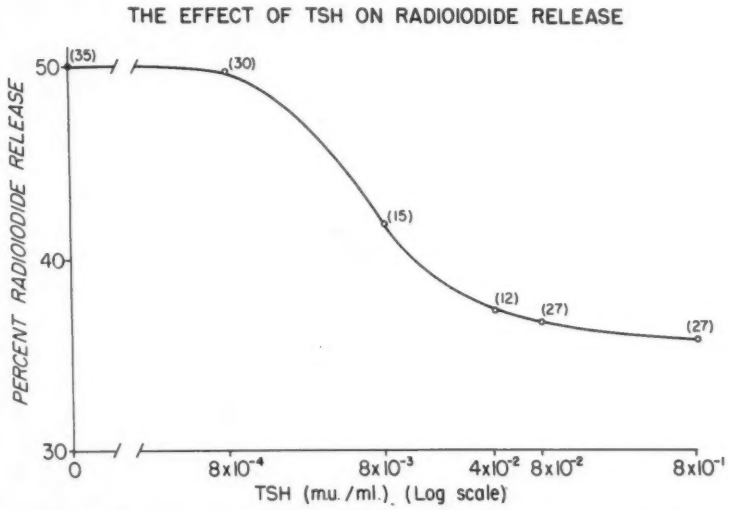

FIG. 6. TSH dose-response relationship as measured by per cent radioiodide release. Details in text. Number of slices from several glands incubated individually per point is stated in brackets. The differences between 8×10^{-4} and 8×10^{-3} m.u. TSH/ml. and between 8×10^{-3} and 8×10^{-2} m.u./ml. are both statistically significant ($P < 0.001$).

uptake period in radioiodide medium, 4 ml./100 mg. of tissue, containing 10μg.% carrier iodide, followed by a four hour release incubation in non-radioactive medium, 10 ml./100 mg. of tissue. The effects of five concentrations of TSH, present in both uptake and release periods, are plotted as a dose-response curve in Figure 6. A TSH concentration of 8×10^{-4} m.u./ml. was not detectable but 8×10^{-3} m.u./ml. significantly ($P < 0.001$) decreased radioiodide release; i.e., increased radioiodide retention. There were statistically significant differences ($P < 0.001$) between the effects of the three concentrations; 8×10^{-4} m.u., 8×10^{-3} m.u. and 4×10^{-2} m.u. of TSH. Higher concentrations of TSH did not further increase the response.

DISCUSSION

Since it is desirable to measure the effects of TSH as directly as possible, it is of importance to determine the earliest and most sensitive measurable response to TSH. Rawson (1949), studying chicks, reported that the earliest and most direct action of TSH was the stimulation of thyroid hormone discharge. This effect could be detected 3–6 hours after TSH injection but were not maximal until 24 hours. Halmi, *et al.* (1953), found that the earliest stimulation of the iodide concentrating mechanism occurred 8 hours after injecting TSH, however PTU blockade was not established in these experiments until one hour before death, i.e., at least 7 hours after the injection of TSH. Tala (1953) observed an increase in both nuclear volume and epithelial percentage in thyroids removed two hours after injecting TSH into guinea pigs. De Robertis (1942) reported an increase in intracellular colloid droplets within 15 minutes after injecting TSH into guinea pigs. We (Bakke and Lawrence, 1954, a,b,c,d) had observed that TSH added *in vitro* caused an increase in thyroid slice QO_2 and organic binding of radioiodine within 30 minutes, and an increase in radioiodide uptake within one hour.

In spite of careful application of some of these observations, the assay of TSH has remained a difficult problem. It may be possible that the *in vitro* radioiodide release method described in this report will contribute to the development of a useful assay. It is reasonable that TSH increases the ability of the thyroid slice to concentrate and retain radioiodide, however in detail, the method appears to be empirical, variable and in its present form lacks a useful range of proportional response. It should be possible to adapt the present technique to estimate the amount of TSH in a fluid sample by using serial dilutions. However, it is hoped that the method can be improved to overcome these shortcomings since it is objective, simple, rapid and probably qualitatively more sensitive to TSH than any previously reported system of detection.[9]

Although both radioiodide uptake and release experiments are probably two different ways of measuring a single biological process, the release experiment proved to be superior. This superiority may be the consequence of the plateau or steady state obtained in the measurement of radioiodide release. Further analysis of this aspect of radioiodide uptake and release kinetics involves a complex mathematical analysis using a polynomial of several exponentials. Obtaining data for this kind of investigation is unusually accessible with slice experiments and will be the subject of a separate report.

[9] It is difficult to make comparisons between reported assays because of an uncertainty about the equivalence of standards and units. If one accepts the statement (Steelman, 1953) that 20 mg. of the standard ox pituitary powder is one U.S.P. unit or 12 J–S (Junkman-Schoeller) units, then DeRobertis (1944) measured 0.16 m.u., D'Angelo, Savano and Gordon (1950) detected 0.17 m.u. or 0.8 m.u./ml. (using Donaldson's statement (1955) that the Parke, Davis 50P4 powder which they used contained 2.U.S.P. units/mg.), Gilliland and Strudwick (1953), measured 3 m.u.; Piotrowski, Steelman and Koch (1953), 12 m.u.; and Crooke and Mathews (1953), 2 m.u.

SUMMARY

Using surviving beef thyroid slices, the collection and release of inorganic radioiodide was measured *in vitro*. The per cent of medium radioiodide collected *in vitro* was independent of the medium carrier iodide concentration and dependent upon the medium volume over a wide range. TSH, added *in vitro*, stimulated the uptake and decreased the release of radioiodide by the thyroid slice. The release of radioiodide by the slice was significantly decreased by as little as 8×10^{-3} m.u. U.S.P. of TSH/ml. of medium.

Although this method for detecting TSH is variable and lacks a useful range of proportional dose-response, it is specific, objective, simple, rapid and probably more sensitive than any yet reported, and it offers an advantageous system for the study of TSH effects on thyroid tissue.

Acknowledgments

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EFFECT OF HEPARIN PRETREATMENT ON STRESS-INDUCED LEUKOCYTE CHANGES IN THE RAT[1]

LYLE H. HAMILTON AND JULIUS LOWENTHAL

Department of Physiology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

IT WAS reported by Godlowski (1951, 1952) that heparin in large doses blocked the eosinopenic action of epinephrine, ACTH and cortisone in humans and in dogs. Weissbecker and Schröter (1954) reported that small doses of heparin blocked the stress-producing effects of ACTH in man, but had no effect on the response to epinephrine or cortisone. However, Eseller, Jeanneret and Schoch (1954) could not block the eosinopenia in man following ACTH when either small or large doses of heparin were administered. The present investigation was conducted to re-evaluate these studies and to extend the observation to a study of all the leukocyte cell-types in the rat.

METHODS

Male rats weighing 150–250 gm. were used in the experiment. They were isolated and kept at a temperature of 26–28° C for at least 4 days and were fasted overnight before the experiment. The experiments were conducted at the same time of the day to eliminate the effects of diurnal rhythm on the blood cell count. Leukocyte counts were carried out on control animals (no injections) and on rats 4 hours after the subcutaneous injection of sodium salicylate (20 mg./100 gm. body weight), epinephrine (20 μg./100 gm.), ACTH (20 mg./100 gm.) and cortisone (12.5 mg./100 gm.). A placebo injection of saline was given to an additional group of rats. All agents were administered in a volume of 0.2 ml./100 gm. body weight. Ninety minutes before these injections 3 mg. heparin (equivalent to 300 I.U.) per animal or an equal volume (0.3 ml.) of saline was given subcutaneously.

Leukocyte counts were carried out on blood samples obtained by cardiac puncture on rats anesthetized by the intraperitoneal injection of 40 mg. pentobarbital sodium. One ml. of blood was collected into a siliconed syringe and transferred to a tube containing powdered potassium oxalate. A dried blood smear was made from a drop of blood taken directly from the syringe for differential counting after Wright's stain. Total leukocyte counts were done in duplicate, and the mean values were used for the data. The duplicate counts were required to check within 10%. At least 200 cells were counted in the differential. Monocytes and lymphocytes were grouped together as mononuclear cells and no difference was recorded in the nuclear configuration of neutrophils.

RESULTS

Table 1 presents the mean leukocyte, eosinophil, mononuclear cell and neutrophil values for the control and experimental groups. The probability

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TABLE 1. BLOOD LEVELS FOR TOTAL LEUKOCYTES, EOSINOPHILS, MONUNUCLEAR CELLS AND NEUTROPHILS IN CONTROL ANIMALS AND IN EXPERIMENTAL GROUPS 4 HOURS AFTER FINAL INJECTION

Probability values (P) are presented for differences between groups pretreated with heparin and saline and for differences between control and experimental groups. P values below 0.05 were considered to indicate significant differences.

Group no.	No. of animals	Treatment	Total leukocytes		Mononuclear cells		Eosinophils		Neutrophils	
			Cells per cmm.	P value for Δ control	Cells per cmm.	P value for Δ control	Cells per cmm.	P value for Δ control	Cells per cmm.	P value for Δ control
I	5	Control (No Injections)	20,440		15,826		326		3,989	
II	8	Heparin + Saline	21,684	0.8 –0.7	17,653	0.7 –0.5	272	0.8 –0.7	3,171	0.5 –0.3
III	10	Saline + Saline	17,912	0.5 –0.3	15,101	0.9 –0.8	161	0.3 –0.2	2,650	0.2 –0.1
P Value for difference between II and III			0.3 –0.2		0.3 –0.2		0.3–0.2		0.5–0.3	
IV	10	Heparin + Salicylate	21,520	0.7 –0.5	17,003	0.7 –0.5	109	0.1 –0.05	4,385	0.8 –0.7
V	16	Saline + Salicylate	14,482	0.05–0.02	10,981	0.05–0.02	105	0.1 –0.05	4,380	0.7 –0.5
P Value for difference between IV and V			0.01–0.001		<0.001		>0.90		>0.90	
VI	11	Heparin + Epinephrine	21,730	0.7 –0.5	13,984	0.5 –0.3	206	0.5 –0.3	7,600	0.05–0.02
VII	11	Saline + Epinephrine	14,834	0.05–0.02	8,288	0.02–0.01	70	0.05–0.02	6,476	0.05
P Value for difference between VI and VII			0.05–0.02		0.05–0.02		0.2–0.1		0.50	
VIII	10	Heparin + ACTH	19,328	0.7 –0.5	14,444	0.7 –0.5	94	0.1 –0.05	4,799	0.7 –0.5
IX	6	Saline + ACTH	10,575	<0.001	7,699	<0.001	46	0.01–0.001	2,851	0.5 –0.3
P Value for difference between VIII and IX			0.01–0.001		0.01–0.001		0.5–0.3		0.2–0.1	
X	18	Heparin + Cortisone	13,159	0.01–0.001	10,226	0.05–0.02	73	0.05–0.02	2,864	0.3 –0.2
XI	10	Saline + Cortisone	12,812	0.01–0.001	9,411	0.01–0.001	93	0.1 –0.05	3,309	0.5 –0.3
P Value for difference between X and XI			0.9 –0.8		0.7 –0.5		0.7–0.5		0.5–0.3	

values for significant differences between groups pretreated with heparin and with saline are presented, as well as the probability values for the significance of differences between the control group and the experimental groups. In all cases a P value ≤ 0.05 was considered significant, i.e., the observed difference was not due to chance. Neither the experimental procedure nor the heparin injection by itself resulted in a change in the leukocyte count, since no significant differences were seen when the untreated group (I) was compared with the group receiving heparin+saline (II) or only saline injections (III). Sodium salicylate, epinephrine, ACTH and cortisone produced significant decreases in the total leukocyte count and mononuclear cell count. Statistically significant decreases were also seen in the eosinophil counts following epinephrine and ACTH. The decreases following salicylate and cortisone injections were of the same order of magnitude as those following epinephrine and ACTH, but were not statistically significant at the 5% level because of larger values for the variance. A significant increase in the neutrophil count was seen only after epinephrine administration. Heparin pretreatment prevented a decrease in the total leukocyte count, mononuclear cells and eosinophils following salicylate, epinephrine and ACTH administrations. It did not prevent the neutrophilia following epinephrine. There were significant differences between heparin and saline pretreatment in the responses of the total leukocyte and mononuclear cell counts following these agents, but the differences in eosinophil and neutrophil counts were not significant. Heparin did not block the decrease in total leukocytes, mononuclear cells or eosinophils following cortisone administration. No differences were seen between the heparin and saline pretreated groups in the total count or in any cell type following cortisone injections.

DISCUSSIONS

The blocking action of heparin on physiological responses to stress was extended in this investigation to include the total leukocyte, mononuclear cell and eosinophil responses in the rat. Heparin inhibited the response following sodium salicylate, epinephrine and ACTH administration, but did not alter the response to cortisone. The significance of the effect of heparin on the eosinophil response in these experiments was probably reduced for 3 reasons: (1) Low values were found in the rats used, (2) It was necessary to use the unpaired-sample technique for the study, and (3) The differential counts were used to calculate the number of leukocyte cell types (indirect method), which further increased the variance. However, when the mononuclear cell response is considered along with the eosinophil response, there can be little doubt that the leukocyte changes reported here were responses to stress. The usually described neutrophilia following stress was significant only in the animals receiving epinephrine.

Weissbecker and Schröter (1954) reported that heparin blocked the action of ACTH on eosinophils, blood sugar and blood pyruvic acid, though

it did not prevent the responses following epinephrine or cortisone. They hypothesized that the heparin inactivated ACTH, rendering it ineffective as a stimulator for the adrenal cortex. In other studies performed in this department, Morris, Lowenthal and Hamilton (to be published) demonstrated that heparin did not prevent adrenal ascorbic acid depletion following different stress-producing procedures. They demonstrated that the adrenal cortex was stimulated in the presence of heparin, probably through the action of ACTH. The possibility exists that heparin interfered with the synthesis or the release of hormones from the adrenal cortex. This was suggested by the observation that only exogenous cortisone was effective in producing a response following heparin administration. However, it has been demonstrated that heparin may play a role in fat transport (Levy and Swank, 1954). Because the solubility characteristics of adrenocortical hormones are similar to those of fat, heparin may have affected their transport in the blood-stream. The injection of cortisone may have been so large that the amount of heparin used was ineffective. Godlowski (1951) demonstrated that large doses of heparin blocked the response to cortisone while Weissbecker and Schröter (1954) and this report indicated that small doses of heparin failed to do so. Investigations of the dose-response relationships between heparin and cortisone are being conducted at the present time.

SUMMARY

The effect of subcutaneous injections of heparin (3 mg.) in rats 90 minutes before the injection of stress-producing agents was studied. The total leukocyte, mononuclear cell and eosinophil counts were reduced 4 hours after the injection of sodium salicylate, epinephrine, ACTH and cortisone. Heparin pretreatment prevented the leukocyte response to sodium salicylate, epinephrine and ACTH, but did not block the response to cortisone in the doses employed in these experiments.

ADDENDUM

Since this paper was submitted for publication it has been shown that the blood cell count response to 5 mg. cortisone per 100 gm. body weight was prevented by 150 I.U. heparin per 100 gm. body weight, while heparin failed to prevent the response to higher doses of cortisone.

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EVIDENCE THAT THE GLOMERULAR ZONE IS STIMULATED BY EPINEPHRINE, CAUSING AN EOSINOPENIA IN HYPOPHYSECTOMIZED RATS

RICHARD A. MILLER AND ALICE W. DOCKRELL[1]
Department of Anatomy, Albany Medical College, Albany, New York

THE experiments reported here concern the number of eosinophils in the peripheral blood of normal rats and of rats in which the well-established pathway by which the pituitary evokes cortical secretory activity was destroyed by hypophysectomy, adrenalectomy or adrenal enucleation, alone and in several combinations, during considerable periods of time. In addition, the changes in the number of eosinophils at one to four hours following single injections of epinephrine or of ACTH at widely spaced intervals into these same animals are reported.

The experiments were undertaken to explore the possibility that the mammalian adrenal cortex may also be stimulated via pathways which do not involve the hypophysis and the release of ACTH. In hypophysectomized pigeons many stimuli, particularly insulin, induce cytologic changes in cortical cells and effect changes in the blood sugar which are indicative of cortical stimulation (Miller and Riddle, 1941, 1942; Riddle and Opdyke, 1947).

MATERIAL AND PROCEDURES

Young adult rats of the inbred Vanderbilt strain were used. Hypophysectomy, adrenalectomy and adrenal enucleation were performed by standard methods. At the time of autopsy, the body was carefully examined under magnification for glandular remnants and no data from rats having a residuum of adrenal or pituitary are included. Although a hypophyseal remnant is readily detected, the method does not exclude the possibility that accessory adrenal cortical tissue might escape detection.

All animals ate *ad libitum* of Wayne Dog Blox. In addition, the operated rats had access to a soft diet containing all necessary nutrients.[2] Hypophysectomized rats drank a 3% aqueous sucrose solution and the adrenalectomized rats a 1.0% aqueous sodium chloride solution, instead of pure water.

Estimations of the number of eosinophils were made by direct enumeration in a Fuchs-Rosenthal counting chamber. The rats were first anesthetized with an intraperitoneal injection of nembutal (0.3 mg per 100 gm. of body weight). Then the freely shed blood from the tail, warmed in water before cutting, was diluted with modification of the

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[2] Lard, 1500 gm.; powdered milk, 1250 gm.; casein, 1250 gm.; wheat germ, 1000 gm.; powdered dog chow, 1000 gm.; yeast powder, 50 gm.; sodium chloride, 46 gm.; cod liver oil, 100 ml.

Randolph eosinophil stain (Roche *et al.*, 1950). The numbers of eosinophils on both tables of the chamber were counted. In determining the average decrease of eosinophils, any increase was recorded as zero and included in the calculation. If the algebraic sum of all changes had been used, very large increases of eosinophils, sometimes observed, would have obscured the eosinopenia which occurred in a majority of the rats. The method for measuring nucleolar size has been published in detail (Miller, 1954).

The procedures used varied only as to details. In general, the eosinophils of six to eight rats, equally distributed as to sex, were estimated at intervals at least a week apart. The initial sample of blood was drawn as quickly as possible after first disturbing the animals and again at one, two, four and usually six hours after the initial sample. The number of eosinophils was determined initially and at similar intervals after the injection of saline to provide control data, and after the injection of epinephrine, ACTH or desoxycorticosterone acetate (DCA).[3] The same animals were used repeatedly during the course of several weeks but never more often than once a week. Many of the groups of intact rats subjected to various tests were then adrenalectomized, enucleated and/or hypophysectomized and tested again for the response to epinephrine or to ACTH.

RESULTS

The normal eosinophil count during extended observation, and the effect of several chronic experimental conditions upon the eosinophil count. The average number of eosinophils in 243 determinations among 68 rats during five weeks was 106, while in 65 additional rats the average of single determinations was 97 (Table 1). The number of eosinophils in the groups was fairly constant from week to week, although there were notable exceptions. Thus, the average eosinophil count was appreciably greater the first time than the second in seven of twelve groups of the intact rats, two groups did not change and in three groups eosinophils were increased slightly in the second count. After either hypophysectomy or adrenalectomy alone, there was no statistical significant change of eosinophils, although the averages suggested a transitory eosinophilia during the fourth and fifth weeks (Table 1). In contrast, a significant eosinopenia persisted during eight weeks after adrenal enucleation. After these same rats were then hypophysectomized, the number of eosinophils increased to that observed in intact or hypophysectomized controls. Quite different from these minimal changes, after concomitant hypophysectomy and adrenalectomy or adrenal enucleation, a most pronounced eosinophilia developed in the first week after operation and persisted during four to six weeks.

Among intact rats injected daily or twice daily with epinephrine, eosinopenia was greatest during the first week of injections, but the eosinopenia was statistically significant during the first three weeks (Table 1). Thereafter the number of eosinophils was normal. By contrast with this prolonged eosinopenia associated with daily administration of epinephrine, an eosinophilia developed during daily injection of DCA and persisted for

[3] Dr. Edward Henderson graciously supplied the desoxycorticosterone acetate (DCA) Schering. We are indebted to Dr. Robert W. Bates for the Squibb corticotrophin (ACTH).

about five weeks. Thereafter the eosinophils reverted to normal, although injections were continued for two more weeks. After injections of DCA were stopped, the number of eosinophils diminished gradually during five succeeding weeks.

Change in number of eosinophils in intact and operated rats one to four hours after a single injection of epinephrine. The effect of 80 μg. of epinephrine per 100 gm. of body weight upon the number of eosinophils at two to four hours after injection was measured in 228 tests upon 57 hypophysectomized rats during nine weeks after operation. The average eosinopenia of all of these measures was 27%, in contrast to the 19% eosinopenia for

TABLE 1. THE NUMBER OF EOSINOPHILIC LEUCOCYTES DURING EXTENDED PERIODS OF OBSERVATION IN NORMAL RATS AND IN RATS SUBJECTED TO A VARIETY OF CHRONIC EXPERIMENTAL CONDITIONS

Weeks										Mean and standard error	t[1] Test
1	2	3	4	5	6	7	8	9	10		
Normal											
123(68)[2]	81(64)	94(56)	111(33)	130(22)						106 ± 7.3	
Hypophysectomized											
124(40)	95(55)	106(50)	140(38)[3]	142(24)[3]	88(12)	57(10)	95(14)			113	
Adrenalectomized											
86(8)	67(8)	122(6)	105(8)	303(4)	91(4)	120(3)				117	
Enucleation of adrenals, later hypophysectomized											
114(5)	60(5)	79(5)	39(5)	57(2)	74(5)	100(4)	38(2)			74 ± 12.0	2.5
88(5)[4]		171(5)						105(5)	59(5)	106	
Simultaneous hypophysectomy and adrenal enucleation											
672(4)	382(12)	413(11)	509(7)	619(6)	792(6)					518 ± 44.0	9.2
Simultaneous hypophysectomy and adrenalectomy											
142(19)	189(19)	301(18)	310(2)							212 ± 31.5	3.3
Normal, injected daily or twice daily with epinephrine											
30(8)[5,6]	59(12)[6]	59(12)[6]	80(12)	110(12)	137(7)	106(4)	98(4)	103(4)		75	
Normal during daily injections of DCA, and after injections were ended											
131(36)	240(35)	195(35)	140(23)	151(17)	110(11)	106(6)				169 ± 13.4	4.2
	156(6)[7]	83(5)	67(5)	58(5)						94 ± 20.8	0.6

[1] t test for the significance of differences between means. Values in excess of 2.0 are considered significant. Experimental groups are compared with normal.
[2] Figures in parentheses represent the number of measurements of eosinophils made.
[3] The t value in a comparison of eosinophils of weeks four and five together with normal controls was 1.3.
[4] Eosinophil counts after the rats were hypophysectomized.
[5] Twenty-four determinations of eosinophils in 10 of these 12 rats before beginning the injection of epinephrine gave an average value of 78.
[6] The t value in a comparison of the eosinophils before and during the first three weeks of daily epinephrine was 2.2.
[7] Values after daily injections of DCA were terminated.

hypophysectomized controls injected with saline (Table 2). The t value in Fischer's test for the significance of difference between these means was 2.1. Thus, although hypophysectomy greatly impairs the response to epinephrine (the standard dose of epinephrine caused a 60% eosinopenia in intact rats, Table 2), it does not abolish the eosinopenic response.

Individual tests of the response to epinephrine in hypophysectomized rats varied between a 400% eosinophilia and a 100% eosinopenia. The several groups of rats displayed wide variability. Groups 1 and 6 of Table 2 showed a marked eosinopenia in repeated tests, while others (group 4) never responded to epinephrine. Usually all or none of the rats of a group

responded or failed to respond to epinephrine. A reason for this variability of response was not established. However, there was direct correlation between the initial number of eosinophils and the degree of diminution of eosinophils.

The eosinopenic response to epinephrine persisted during nine weeks after hypophysectomy. In contrast, the eosinopenic response to ACTH was impaired or abolished as the period after hypophysectomy increased (Table 3). Similar observations are reported by Sayers *et al.* (1944).

TABLE 2. PER CENT DECREASE IN THE NUMBER OF EOSINOPHILIC LEUCOCYTES AT TWO OR FOUR HOURS AFTER AN INJECTION OF EPINEPHRINE REPEATED AT WEEKLY INTERVALS

No. of rats	Weeks									Average
	1	2	3	4	5	6	7	8	9+	
					Normal control					
18	20	—	16	6	12					15±3.0(48)
					Hypophysectomized control					
10	20	16	21	17						19±3.5[1](33)
					Normal and epinephrine[2]					
19	55	56(5)[3]	72(5)							60(28)
					Hypophysectomized and epinephrine					
4	38	32	45	9	16	41	50	34		
2	17	0	35	0	47	42	14	—	25	
6	46	60	—	70(4)	—	—	10(4)	18(4)		
8	—	8(7)	3	14	1					
4	59	50	23	12(2)	46(2)					
6	34	28	29	43						
8	6	19	35(6)	25(6)						
5	32	13	—	28						
8	41	52	32							
6	37	13	25							
Average	31(49)	28(56)	24(44)	27(37)	16(16)	42(6)	18(10)	26(8)	25(2)	27±2.0(228)[4]
					Adrenalectomized and epinephrine					
10	23	22(8)	15(8)	19(8)	36(4)	15(5)	39(2)			22±3.4(45)[5]
					Enucleation of adrenals, and epinephrine later hypophysectomy					
5	47	36	59	62	82	39				54(30)
	17	—	0	—	—	—	—	27	20(4)	16(19)
					Simultaneously hypophysectomy, adrenal enucleation and epinephrine					
16	8(12)	38(16)	23(14)	37(18)	33(7)	36(1)	11(2)			27±3.2(60)[6]
					Hypophysectomy, adrenalectomy and epinephrine					
25	12	13(18)	22(12)	22(7)						15(62)

[1] Standard error of mean.
[2] A standard dose of 80 μg. per 100 gm. of body weight was used in all experiments.
[3] The figures in parenthesis are the number of determination of eosinophils. Where not stated, the number of determinations and the number of rats used are the same.
[4] the *t* test in comparison with the hypophysectomized controls is 2.1.
[5] The *t* test in comparison with normal controls was 1.3.
[6] The *t* test in comparison with the hypophysectomized controls was 1.6.

Epinephrine did not evoke a significant eosinopenia in adrenalectomized rats (Table 2), although individual rats might develop a considerable eosinopenia, i.e. 36%. If adrenal rests occurred in such animals, they could be stimulated by epinephrine via the hypophysis. In support of this view, among hypophysectomized and adrenalectomized rats injected with epinephrine the decrease of eosinophils was within the range of intact and hypophysectomized controls (Table 2).

Removal of the fascicular zone by enucleation (and the reticular zone and medulla) did not abolish the response to epinephrine. If the hypophysis

TABLE 3. PER CENT DECREASE IN THE NUMBER OF EOSINOPHILIC LEUCOCYTES AT INTERVALS AFTER A SINGLE INJECTION OF ACTH INTO RATS, HYPOPHYSECTOMIZED FOR DIFFERENT PERIODS OF TIME

Number of rats	Hours after injection				
	1	4	7	10	13
	Hypophysectomized 1 week, 1 I.U. per 100 g. of body weight				
6	8	3	10	31	15
	Hypophysectomized 2 weeks, 3 I.U. per 100 g. of body weight				
8	28	2	25	39	
	Hypophysectomized 5 weeks, 3 I.U. per 100 g. of body weight				
10	25	14	2	1	1
	Hypophysectomized 9 weeks, 3 I.U. per 100 g. of body weight				
4	8	26	0	0	0

was intact, eosinophilia was similar to that in normal injected rats, while after simultaneous hypophysectomy and adrenal enucleation the eosinopenic response to epinephrine was identical to that of hypophysectomized, injected rats.

Cytological phenomena in adrenals of hypophysectomized rats injected with epinephrine. An earlier study (Miller, 1954) demonstrated that the nucleolus, and to a limited extent the nucleus, reflects the secretory state of adrenal cortical cells by changes in size. The procedures established in that study were employed to measure the change in the nuclei of hypophysectomized rats at intervals between one and ten hours after a single injection of epinephrine. While epinephrine had no effect upon nucleolar and nuclear size in cells of the fascicular zone, glomerular nucleolar size increased and was significantly enlarged ten hours after injection (Table 4).

TABLE 4. THE SIZE OF CAMERA LUCIDA TRACINGS OF THE NUCLEOLUS AND NUCLEUS IN THE GLOMERULAR AND FASCICULAR ZONES OF HYPOPHYSECTOMIZED RATS AT INTERVALS AFTER ONE INJECTION OF EPINEPHRINE

	No. of rats	No. of cells	Nucleolus		Nucleus	
			Glomerular Zone	Fascicular Zone	Glomerular Zone	Fasciular Zone
Hypophy. Control	14	140	26.7 ± .3[1]	31.5 ± .7	603	688
1 hour	9	90	26.4	33.1	594	713
4 hours	13	130	26.7	31.5	575	680
7 hours	10	100	28.0	33.3 ± 8[3]	595	695
10 hours	7	70	30.2 ± .82[2]	31.0	605	639

[1] Standard error of mean.
[2] The *t* value in comparison with the control was 3.9.
[3] The *t* value in comparison with the control was 0.7.

DISCUSSION AND CONCLUSIONS

The present study demonstrates that several chronic experimental conditions which affect the adrenal cortex also effected an eosinophilia (DCA) or an eosinopenia (epinephrine, adrenal enucleation). Excepting adrenal enucleation, although the experimental modification of eosinophils was prolonged, reversion to normal occurred after three to six weeks of the

experimental procedure. Cytologically, DCA inhibits both glomerular and fascicular zones but epinephrine stimulates only the fascicular zone of intact animals (Miller, 1950, 1953, 1954).

It is notable that neither adrenalectomy nor hypophysectomy alone evoked a statistically significant permanent change in eosinophils. Jakobson and Hortling, 1954, observed a transitory eosinophilia after adrenalectomy and one of four weeks duration after hypophysectomy. Others report eosinophilia after hypophysectomy or adrenalectomy (Stein and Martin, 1951; Speirs, 1953), but the observation is not general (Speirs and Meyer, 1949; Recant, *et al.*, 1950).

In contrast to the present failure of either hypophysectomy or adrenalectomy to evoke a significant change of eosinophils (and to the moderate and impermanent effects of daily DCA, epinephrine or adrenal enucleation) the combined removal of both the hypophysis and adrenals, either wholly or by enucleation, evoked a pronounced eosinophilia which persisted unabated throughout periods of four and six weeks. These observations suggest that even in the absense of the hypophysis, the adrenal exercises a check upon the level of eosinophils of the peripheral blood. However, the glomerular zone alone did not exercise such a check, since the number of eosinophils was greatest in rats which were both hypophysectomized and adrenal enucleated.

Several lines of evidence indicate that after hypophysectomy it is the glomerular zone which is stimulated by epinephrine to cause the eosinopenia occurring two to four hours after injection. First, the eosinopenic response to epinephrine is the same in hypophysectomized rats whether the adrenals are intact or have been enucleated. Second, although the adrenal becomes unresponsive to a single injection of ACTH about two weeks after hypophysectomy, there is no diminution in the degree of eosinopenia induced by epinephrine for as long as nine weeks after hypophysectomy. Finally, there is cytologic evidence of progressive nucleolar hypertrophy in the glomerular zone between one and ten hours after injecting epinephrine. Nucleolar hypertrophy and atrophy have been correlated with secretory activity and inactivity in adrenal cortical cells (Miller, 1954).

It thus seems probable that epinephrine can evoke secretory activity in the adrenal either directly (in the absence of the pituitary) or indirectly through the mediation of the hypophysis. It is further probable that it is the glomerular cells which are directly stimulated by epinephrine rather than fascicular cells.

SUMMARY

The effect of several experimental conditions upon the number of eosinophilic leucocytes during prolonged periods and the capacity of epinephrine to cause an eosinopenia at two to four hours after injection into rats were investigated. Experimental conditions included hypophysectomy, adre-

nalectomy, adrenal enucleation, alone and in combination, and daily injections of desoxycorticosterone acetate (DCA) or epinephrine.

Hypophysectomy or adrenalectomy did not change significantly the number of eosinophils. Daily injections of epinephrine resulted in an eosinopenia of three weeks duration, while DCA caused a slight eosinophilia during several weeks. None of these changes of eosinophils was permanent. However, when rats were both hypophysectomized and adrenalectomized or adrenal enucleated, eosinophilia was pronounced and persisted unabated up to six weeks after operation.

In contrast with these chronic changes, epinephrine caused an eosinopenia at two to four hours after injection into hypophysectomized rats or into hypophysectomized and adrenal enucleated rats. This acute eosinopenia after epinephrine did not occur when rats were simultaneously hypophysectomized and adrenalectomized. Experimental and cytologic evidence is presented which indicates that it is the glomerular zone which is stimulated by epinephrine in the absence of the anterior pituitary gland to mediate the eosinopenia.

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ZONE ELECTROPHORESIS IN THE PREPARATION OF SERUM AND PITUITARY EXTRACTS FOR BIOASSAY OF THYROID STIMULATING HORMONE[1]

SHOLEM POSTEL[2]

Medical Services of the Massachusetts General Hospital and the Department of Medicine of the Harvard Medical School, Boston, Massachusetts

THE study of thyroid gland disease has been hampered by technical obstacles to measurement of thyroid stimulating hormone (TSH) in human blood. Application of several bioassay techniques, including the very sensitive tadpole method of D'Angelo, has so far yielded fragmentary or contradictory data (Hertz and Oastler, 1936; Fellinger, 1936; DeRobertis, 1948; d'Angelo *et al.*, 1951; Asboe-Hansen *et al.*, 1952; Gilliland and Strudwick, 1953; Simkin *et al.*, 1953; Adams and Purves, 1953; Sampson *et al.*, 1954). Most of these bioassays have been made on whole serum without pretreatment aimed at isolation of the TSH activity. The importance of isolating the active fraction was emphasized by Fellinger (1936) who found that serum TSH effects were more than doubled by acetone precipitation of hemolyzed whole blood and ascribed the augmented TSH biopotency to the elimination of thyroxin. Similarly, DeRobertis (1948) observed no detectable TSH activity in blood prior to acetone precipitation.

The studies described in the present report refocus on the concept that circulating TSH is obscured by serum constituents which interfere with its bioassay and that isolation of the TSH might permit its detection by methods more simple, though less sensitive, than the stasis tadpole bioassay (D'Angelo, 1951). Serum was fractionated by preparative zone electrophoresis and the fractions bioassayed using radioactive iodine (I^{131}) uptake by the chick thyroid gland as indicator of TSH activity. The conditions of the electrophoretic separation were guided by preliminary observations of the transport properties of the TSH activity in beef pituitary preparations and in human crude pituitary extract. In addition, the method was examined for its ability to separate TSH from the iodine containing fraction of serum and to recover TSH from an *in vitro* system in which its effects were masked by thyroxine.

METHODS

(A) *Preparative zone electrophoresis*

Electrophoresis of 3–4 ml. of serum and pituitary preparations was performed in a starch-supported medium as described by Kunkel and Slater (1952). Thrice washed

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potato starch[3] was suspended in buffer solution to form a thick paste which was then moulded in a plastic trough. The test preparation was injected into a slit formed in the block with a spatula. In all experiments the buffer was 0.04 M sodium phosphate, pH 7.4, of ionic strength 0.10. Two two-liter beakers bridged by an inverted U-tube formed the buffer assembly at each end of the block. Voltages of 300 to 600, yielding currents of 30–50 milliamperes, were applied for 16–22 hours. Room temperature was maintained at 1–3° C. Segments of the block of 1 cm. width were resuspended in buffer solution, stirred, and the supernatant decanted for bioassay and other determinations after the starch particles settled. Aliquots of eluates from two to four segments were pooled for bioassay.

(B) *Chemical determinations*

Protein concentration of eluates was determined by the Folin tyrosine method as modified by Lowry *et al.* (1951) using a crystalline bovine serum albumin standard. Sodium and potassium were estimated by flame photometry, chloride by the method of Wilson and Ball (1928) and total lipids by the Sudan black method described by Swahn (1953). pH and oxidation-reduction potentials were measured with the Beckman model G pH meter.

(C) *Assay of thyroid stimulating activity*

Assay of thyroid stimulation was based upon I^{131} uptake by the chick thyroid. The method derives from Rawson and Salter's (1940) observations of the suitability of day-old chicks for bioassay and is patterned after the early I^{131} experiments of Keating *et al.* (1945).

Red-rock sex-linked hybrid cockerels,[4] received on the day of hatching and maintained at brooder temperatures of 85–95° F, were given tap water and a chick starter mash[5] containing 1.6 μg. iodide per gram. Volumes of 1.0 ml. of test and control solutions were injected subcutaneously on the day of hatching and at 24-hour intervals thereafter for a total of 3 doses. Eighteen hours after the last injection 0.2 μc. of carrier-free I^{131} was administered intramuscularly. Four hours later the chicks were sacrificed by ether inhalation. Radioactivity of their excised thyroid glands was determined in a well type scintillation detector with a sensitivity of one million counts per microcurie. The uptake was expressed as per cent of the administered dose. TSH dose-response curves covered the range of 0.005 to 0.050 U.S.P. units/ml. with each point representing the mean response of 5 chicks. This dose range is represented by an eight- to ten-fold increase in I^{131} uptake.

(D) *Preparations*

All material designated "beef TSH" refers to lot K46808R or P58980 of a lyophilized product[6] of alkaline-alcohol fractionation of beef anterior pituitary powder. The human pituitary extract was prepared from one-half of each of three glands frozen in CO_2 immediately after removal at necropsy. These were pooled, homogenized with a Potter homogenizer in 5 ml. distilled water for one minute and centrifuged. l-thyroxine and l-triiodothyronine were purified by chromatography and recrystallization.[7]

[3] Amend Drug and Chemical Company, Inc., New York, N.Y.

[4] Harco Orchards and Poultry Farms, Inc., South Easton, Massachusetts. When hybrids were not available Red Cockerels were employed.

[5] Purina Chick Startena.

[6] Generously supplied by the Armour Laboratories through Drs. Richard A. Carey and Sanford L. Steelman.

[7] Kindly provided by Dr. Rosalind Pitt-Rivers.

RESULTS

A. *Electrophoresis of bovine TSH*

Zonal analysis of the protein distribution and biological activity after electrophoresis of 10 U.S.P. units beef TSH (#K46808R) showed that thyroid stimulating activity migrated toward the cathode together with the major protein peak (Fig. 1). Two of the anodal pools inhibited I^{131} uptake to 41 and 31%, respectively, of the control value of the chicks to whom only phosphate buffer was given ($P = 0.005$ and 0.005, respectively). Analysis of a second preparation (#P58980) yielded similar results.

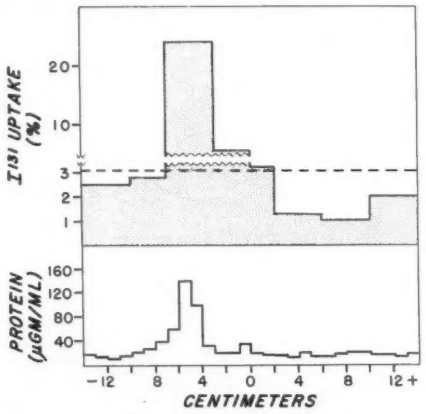

FIG. 1. *Electrophoresis of Beef TSH.* 10 U.S.P. units dissolved in 3 ml. pH 7.4 phosphate buffer; 30 mA current for 20 hours. The lower graph represents protein concentration. The height of the stippled area in the upper portion of the figure indicates the biological response to each eluate pool expressed as 4 hour I^{131} uptake in per cent. The mean uptake by the control chicks is indicated by the broken horizontal line. TSH activity peaks in pool -4 through -7. I^{131} uptake is depressed in pools $+3$ through 6 and $+7$ through 10.

B. *Electrophoresis of bovine TSH in presence of human serum*

To test whether the stimulating and inhibiting zones disclosed by fractionation of partially purified beef TSH maintained differential mobility in a more complex biological system an electrophoretic fractionation was performed after incubation for one hour, of 6 U.S.P. units beef TSH with 3 ml. normal fasting human serum. Thyroid stimulating activity concentrated in the -5 through -7 zone as it did in the absence of serum, but there was considerable proximal "smearing" (Fig. 2). The peak of activity corresponded to the descending limb of the serum γ-globulins. Again, in anodal pools there was suppression of the end point to values approximately one-half those of controls ($P = 0.02$, 0.005, and 0.01, respectively, for the differences between I^{131} uptakes of 3.8%, 3.6% and 3.9% in pools

+6 through 8, +9 through 11, and +12 through 14 and the control uptake of 6.6%). The inhibitory action of these anodal segments was confirmed by testing their influence on *added* TSH. Beef TSH was added to aliquots of the original inhibitory eluates to make a final TSH concentration of 0.02 U.S.P. units/ml. Interference with the effect of added TSH by the inhibitory pools was shown by I^{131} uptakes of 16.6%, 13.9% and 17.9% in response to pools +6 through 8, +9 through 11, and +12 through 14, respectively, compared with the control value of 22.4% for chicks receiving TSH alone ($P = 0.2$, 0.025, and 0.3 respectively).

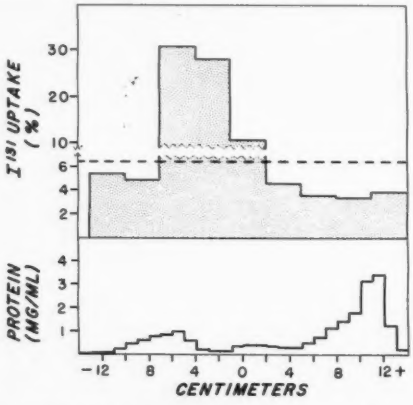

FIG. 2. *Electrophoresis of Beef TSH in Presence of Human Serum.* 6 U.S.P. units incubated with 3 ml. normal human serum for one hour. 30 mA current for 20 hours. Key as in Fig. 1. A zone of inhibition of uptake is seen similar to that in Fig. 1.

C. *Electrophoretic separation of exogenous TSH from serum in vivo labeled I^{131} fraction and thyroxine*

Serum obtained from a thyrotoxic patient 5 days after treatment with 7 millicuries of I^{131} was mixed with TSH and thyroxine to give concentrations of 0.8 U.S.P. units of TSH per ml. and 240 μg. l-thyroxine per ml. Bioassay of this mixture yielded a TSH potency equivalent to only 0.24 U.S.P. units per ml., i.e., 30% of the actual TSH present. Electrophoresis of 3 ml. of the same serum-thyroxine-TSH mixture revealed distinct separation of the *in vivo* labeled I^{131} from the TSH containing fractions (Fig. 3). Measurement of the *total* TSH activity in the eluates accounted for 2.0 U.S.P. units, or 0.68 U.S.P. units per ml. of the original mixture. This corresponds to an 85% recovery of TSH compared with the pre-electrophoresis value of 39%. The suppression of uptake in pool +12 through +14 is due to the concentration of the added thyroxine in this zone. Inhibition was also observed in pool +9 through +11. Since 6% of the labeled

iodine was present in the latter pool it is not certain whether the inhibition observed here is due to a corresponding amount of thyroxine trailing into it or to the inhibitor in the TSH preparation itself.

D. *Electrophoresis of human crude anterior pituitary extract*

In order to demonstrate whether the thyroid stimulating fraction of human and bovine pituitaries behaved similarly, a crude extract of human anterior pituitary was examined. The protein pattern (Fig. 4) was qualitatively that displayed by serum electrophoresed under these conditions. The thyroid stimulating activity was associated with the major peak and a distinct inhibitory zone represented by the pool of segments +6, 7, 8 was

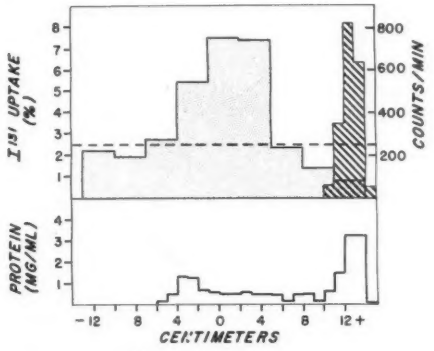

FIG. 3. *Electrophoresis of a TSH-Thyroxine-Serum Mixture.* 3 ml. of serum from a thyrotoxic patient 5 days after I^{131} treatment. 240 μg./ml. l-thyroxine and 0.8 U.S.P. units beef TSH/ml. were introduced one hour before the electrophoresis was begun. 40 mA current for 16 hours. Key as in previous figures with the addition of cross hatching to denote the amount of I^{131} in each eluate in counts per minute. Separation of TSH activity from the labeled iodine containing fraction is apparent.

clearly demonstrated (Fig. 4). The uptake by chicks under the influence of this zone is 1.1%, i.e., only 27% of the control value of 4.1% ($P = <0.02$).

E. *The biological activity of human sera fractionated by zone electrophoresis*

Sera of 6 subjects, 1 normal, 1 with chronic thyroiditis and post-operative myxedema, and 4 with thyrotoxicosis were bioassayed before and after zone electrophoresis in an attempt to uncover fractions with thyroid stimulating activity. None of the untreated sera or 54 eluate pools obtained by electrophoresis contained such activity. To test for an inhibitor of the action of TSH, the eluates were also bioassayed after the addition of beef TSH usually in a final concentration of 0.02 U.S.P. units/ml. In the sera of the normal subject, the myxedema patient and two of the patients with thyrotoxicosis there was partial to complete interference with the efficacy

of the added hormone in one or more eluates. An example of this effect is shown in Figure 5 in which the location of the inhibitory zone corresponds to the inhibiting fractions observed in the TSH preparations and the human crude pituitary extract.

In two of the thyrotoxic patients the serum analyses were made 6 days after treatment with radioactive iodine in order to localize labeled iodine compounds in relation to the inhibitor zones. The fractions which contained the radioactivity permitted full biological activity of added TSH. This observation and the finding of inhibitory zones in myxedema serum are evidence that thyroid hormones are not involved in the inhibitory phenomena.

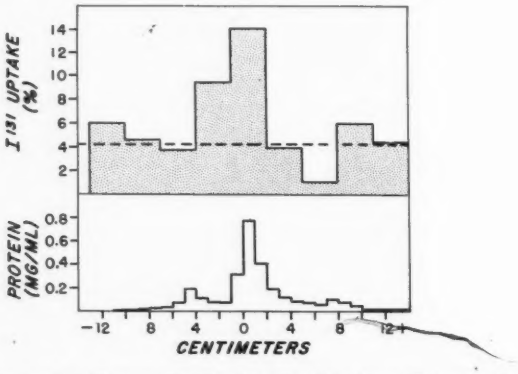

FIG. 4. *Electrophoresis of a Human Crude Anterior Pituitary Extract.* 40 mA current for 21 hours. Key as in Fig. 1. In addition to TSH activity, a distinct inhibitory zone corresponding to those of Figs. 1 and 2 is shown in the pool of segments +6 through 8.

F. *Control studies*

In an attempt to appraise the significance of the observed inhibitory phenomena various factors which might influence the efficacy of TSH or I^{131} uptake by the thyroid were investigated. Since the protein content of eluates was variable the influence of protein was assessed by comparing 5 point dose-response curves for bovine TSH in vehicles of 0.9% NaCl, 0.04 M pH 7.4 phosphate buffer, a 1:20 dilution of serum, and a 0.3% phosphate buffered crystalline bovine albumin solution. These showed no significant differences. Similarly, incubation of TSH with starch did not modify its potency.

In two experiments the efficacy of TSH added to eluates of electrophoresed serum (in which inhibitory zones had been found) did not vary with small differenses in pH, oxidation-reduction potential or with the distribution of serum lipids or salts.

Data on TSH effects in the presence of simultaneously administered thyroxine and triiodothyronine were obtained. Fifty per cent or greater suppression of the unstimulated chick thyroid gland uptake occurred with three injections of 1.29×10^{-9} moles of thyroxine or triiodothyronine (Table 1). Comparable inhibition of the response to 0.020 U.S.P. units of TSH required 1.29×10^{-8} moles of thyronine compound or ten times the

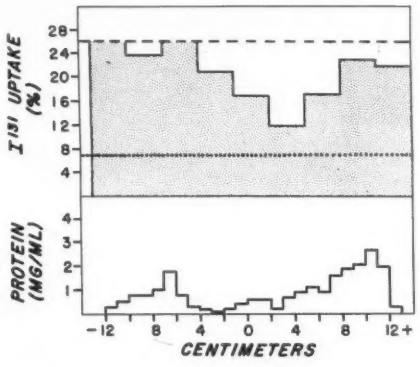

FIG. 5. *Electrophoresis of Myxedema Serum.* 3 ml. serum from female subject with chronic thyroiditis and postoperative myxedema. 40 mA current for 21 hours. Eluates from 1 cm. segments were pooled in groups of three. Beef TSH solution was added to each of the nine pools so as to achieve a final TSH concentration of 0.02 U.S.P. units/ml. The height of the stippled area indicates 4 hour I^{131} uptake by chicks receiving the eluate-TSH-mixture. The broken horizontal line at 25.3% represents the uptake of control chicks receiving 0.02 U.S.P. TSH units/ml. and the dotted line at 7.5% represents the mean uptake of chicks receiving no TSH. Suppression of the response to TSH added to the eluates is maximal in pool +3 through +5.

TABLE 1. THE INFLUENCE OF l-THYROXINE AND l-TRIIODOTHYRONINE ON THE RESPONSE TO EXOGENOUS TSH

Dose of thyronine compound[2] (moles)	I^{131} uptake[1]					
	0 TSH		0.004 U TSH		0.020 U TSH	
	T_4	T_3	T_4	T_3	T_4	T_3
0	9.6 (1.7)	9.6 (1.7)	14.2 (5.1)	14.2 (5.1)	42.6 (1.6)	42.6 (1.6)
1.29×10^{-10}	8.3 (1.1)	7.2 (2.2)	15.0 (6.4)	15.3 (4.8)	39.3 (8.5)	33.2 (10.8)
1.29×10^{-9}	4.8 (1.0)	4.7 (1.6)	14.3 (3.6)	15.6 (5.1)	35.3 (5.6)	30.1 (10.3)
1.29×10^{-8}	2.1 (1.0)	2.5 (0.7)	1.8 (0.9)	5.2 (1.3)	16.9 (5.7)	25.9 (7.7)

[1] The mean uptake by the thyroids of 5 chicks expressed in per cent. The standard deviation of the sample is in parentheses.
[2] The expressions T_4 and T_3 refer to l-thyroxine and l-triiodothyronine, respectively. The designated doses of thyronine compound and TSH were administered on each of three consecutive days.

quantity required to suppress resting gland uptake (Table 1). On the basis of the above data it was calculated that the amounts of thyroxine or triiodothyronine needed to account for the inhibitory phenomena observed in the pituitary preparations and sera exceed the serum concentration of total organic iodine by a factor of at least one thousand.

DISCUSSION

It is apparent from these studies that the electrical mobility of exogenous thyroxine and the *in vivo* I^{131} labeled serum fraction differs from that of beef TSH. The mobility difference allows clear cut isolation of beef TSH either from thyroxine added to serum, or from the *in vivo* labeled serum I^{131}.

These observations conform to expectations based on many studies of serum transport of thyroxine or protein-bound I^{131} (Gordon *et al.*, 1952; Larson *et al.*, 1952; Horst and Roesler, 1953; Robbins and Rall, 1952; Dingledine *et al.*, 1955), and reports that beef TSH contains a strong basic group and has an isoelectric point in the pH 8 to 9 range (Steelman *et al.*, 1952; Heideman, 1953; Fels *et al.*, 1955). Although electrophoretic identity of beef and human thyroid stimulating substance remains to be shown by more discriminating techniques, it appears from the examination of the human crude pituitary extract that human pituitary TSH, like beef pituitary TSH, is associated with proteins which, by virtue of their relatively high isoelectric points, can be separated from the thyroxine carrying fractions.

It is clear that *in vitro* masking of TSH effect by administration of thyroxine can be reversed by electrophoresis. This indicates that inhibition of TSH effects by thyroxine, either by a direct effect at thyroid cell level (Cortell and Rawson, 1944) or by promoting accelerated removal of circulating TSH (D'Angelo, 1955) does not imply structural alteration of TSH by thyroxine. It is therefore assumed that the failure to uncover endogenous serum TSH in these experiments is not due to a direct effect of thyroxine on the biological integrity of TSH.

Despite the demonstration that TSH "neutralized" by thyroxine can be restored by electrophoretic diversion of thyroxine, no thyroid stimulating activity was uncovered by fractionation of 6 sera. This is most likely due to the limit of detectability of endogenous material by this method even after elimination of factors modifying the biological response. It is possible that a more sensitive method of assay such as the stasis tadpole technique (D'Angelo, 1951) would disclose quantities of TSH even in excess of those found in *whole* serum by the tadpole assay.

The demonstration of fractions which inhibit the end point in the bioassay of TSH is of interest in its own right. In addition, it dictates caution in the interpretation of TSH data which may express the interplay of inhibitors and stimulators in the particular bioassay rather than primary physiological events. The nature of the inhibitor substance or substances is unknown. The inhibition by fractions free of I^{131} and the presence of inhibitor in myxedema serum indicate that thyroid hormones are probably not involved. This interpretation is reinforced by the finding that the amounts of thyroxine and triiodothyronine required for the inhibitory ef-

fects observed in these experiments exceed the usual blood concentration of these substances by a factor of one thousand.

The control physico-chemical studies showed that the inhibitor fractions are not artifacts due to changes in pH or oxidation-reduction potential, total lipid, protein concentration or the presence of starch. On the other hand, it is possible that a known pituitary or other endocrine gland product is involved. It has been observed that thyroid uptake of I^{131} in the chick is inhibited by cortisone in the presence or absence of TSH (Shellabarger, 1954). However, the required total cortisone dose of 10–15 mg. per chick implies an impossible concentration of serum cortisone (3500 mg.%). Other normal body constituents, especially non-specific proteolytic or peptidolytic enzymes remain to be evaluated in this connection. It should be noted that the inhibitory phenomena may reflect only one phase of TSH effect, i.e., stimulation of I^{131} uptake.

SUMMARY AND CONCLUSIONS

Using zone electrophoresis in a starch-supported phosphate buffer medium at pH 7.4, and a chick I^{131} uptake assay for TSH, it was shown that:

The thyroid stimulating activity of a partially purified commercial beef TSH preparation is associated with the protein peak and is transported primarily with the γ-globulins of human serum when added *in vitro*.

There is clear cut separation between the I^{131} containing fraction of serum or thyroxine and exogenous TSH.

Inhibition of the manifest efficacy of TSH by thyroxine is reversible by electrophoresis and does not imply structural alteration of the TSH.

The observed differences in transport of beef TSH and thyroxine probably also apply to human pituitary TSH.

Beef TSH preparations and human crude pituitary extract display comparable fractions which inhibit the end point in the bioassay.

Fractionation of human sera reveals a non-iodine containing inhibitor of the augmented uptake of I^{131} in response to beef TSH but does not uncover endogenous TSH.

Acknowledgment

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THE MYOTROPHIC AND ANDROGENIC EFFECTS OF 17-ETHYL-19-NORTESTOSTERONE AND RELATED COMPOUNDS

FRANCIS J. SAUNDERS AND VICTOR A. DRILL
Division of Biological Research, G. D. Searle & Co., Chicago, Illinois

WHILE adequate assay procedures have long been available to determine the androgenic effects of steroids, there was no satisfactory rapid method of assaying their anabolic effects until development of the levator ani technique by Eisenberg and Gordan (1950). This method is based on the fact that after castration the levator ani muscle atrophies and its weight can be restored by treatment with testosterone propionate and other androgenic steroids.

Hershberger, Shipley and Meyer (1953), using a modification of this method, showed that 19-nortestosterone, while exhibiting essentially the same myotrophic activity as testosterone, has only relatively slight androgenic effects. As a measure of the relative activities they used the ratio of the increase in levator ani weight to the increase in ventral prostate weight. The values obtained were about 0.3 for testosterone and 1.0 for 19-nortestosterone.

Stafford, Bowman and Olson (1954), measuring nitrogen retention in force-fed castrated rats, reported that 19-nortestosterone cyclopentylpropionate was between one-half and two times as active as testosterone propionate. The present study is concerned with several compounds related to 19-nortestosterone and their biological activities.

MATERIAL AND METHODS

The assay used to determine androgenic and myotrophic activities was that of Eisenberg and Gordan (1950). White rats of the Sprague-Dawley strain were castrated at 30 days of age. Starting three weeks later, they were injected intramuscularly daily for 7 days with one-seventh of the total dose dissolved in 0.1 ml. corn oil. On the day after the last injection they were sacrificed and the seminal vesicles, ventral prostates and levator ani muscles were removed and weighed. In addition to testosterone propionate and 17-ethyl-19-nortestosterone, other related compounds were studied.

RESULTS

The results of a typical assay involving testosterone propionate and 17-ethyl-19-nortestosterone are shown in Table 1. Eight rats were used in each group. It is clear that the minimal doses required to produce significant increases in levator ani weights are approximately equal, while the

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TABLE 1. THE EFFECTS OF TESTOSTERONE PROPIONATE AND 17-ETHYL-19-NORTESTOSTERONE ON THE ACCESSORY SEX GLANDS AND THE LEVATOR ANI MUSCLE OF CASTRATED RATS

Compound	Total dose	Seminal vesicle	Ventral prostate	Levator ani
	mg.	mg. ±S.E.	mg. ±S.E.	mg. ±S.E.
oil only	—	9.4±0.4	13.4±0.5	55.5±4.1
testosterone propionate	0.05	13.0±0.8	17.4±1.3	73.0±8.0
	0.2	48.0±2.6	43.4±3.4	80.5±7.1
	0.8	128.1±6.6	129.6±5.7	107.2±7.2
17-ethyl-19-nortestosterone	0.2	11.1±0.9	12.5±0.7	78.4±5.2
	0.8	14.5±0.5	16.5±0.9	105.7±6.7
	3.2	32.6±2.4	23.9±2.1	123.4±6.1

androgenic potency of 17-ethyl-19-nortestosterone is only about 6% of that of testosterone propionate. This relative lack of androgenic activity is also shown in the weights of the ventral prostates.

Testosterone propionate and 17-ethyl-19-nortestosterone were further studied in comparison with testosterone. Figure 1 depicts the relative anabolic activities of these compounds, while the androgenic effects are

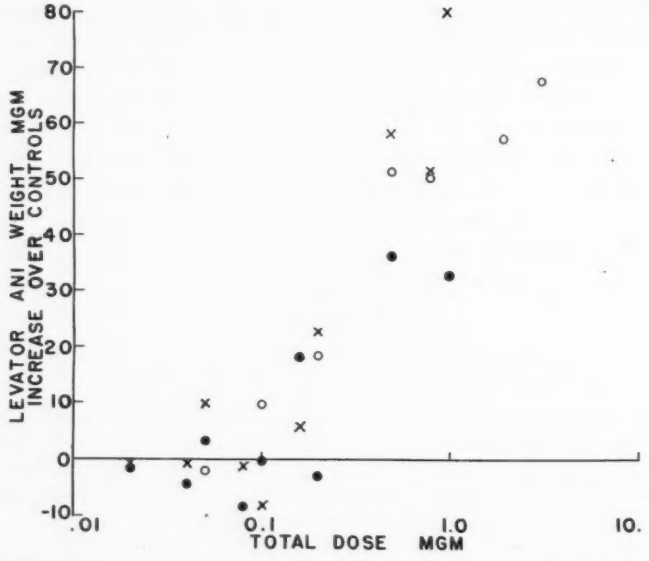

FIG. 1. The effect of several steroids on the increase in weight of the levator ani muscle in castrate rats. Each point represents the difference in average weight obtained in one or more groups of 8 treated animals and 8 controls autopsied on the same day. One-seventh of the total dose was administered intramuscularly each day. The crosses represent testosterone propionate; the solid circles, testosterone; and the open circles, 17-ethyl-19-nortestosterone.

shown in Figure 2. Differences in the slopes as well as differences in minimal androgenic activities are evident. The graphs thus demonstrate that the absolute androgenic activity of 17-ethyl-19-nortestosterone is less than that of either testosterone or testosterone propionate, while the myotrophic potencies are similar.

Estimates of the androgenic and myotrophic potencies of some related compounds are shown, in terms of testosterone propionate, in Table 2. Since the slopes of the curves did not always parallel that of testosterone propionate, minimal doses which produced a significant increase in the weight of the seminal vesicles or levator ani were compared. It is evident that side chains in the 17 position containing more than two carbon atoms, or containing unsaturated linkages, reduce the myotrophic activity.

TABLE 2. ESTIMATED ANDROGENIC AND MYOTROPHIC POTENCIES OF 19-NORTESTOSTERONE DERIVATIES IN CASTRATE MALE RATS

Compound	Androgenic* potency	Myotrophic* potency
testosterone propionate	100	100
19-nortestosterone	6	100
17-methyl-19-nortestosterone	6	100
17-ethyl-19-nortestosterone	6	100
17-propyl-19-nortestosterone	<1	10
17-butyl-19-nortestosterone	<2	<5
17-vinyl-19-nortestosterone	2	20
17-ethynyl-19-nortestosterone	estrogenic**	1
enol acetate of 17-ethynyl-19-nortestosterone 17-acetate	estrogenic**	<2
19-nor-4-androstene-3,17β-diol	5	50

* Potencies are given in terms of per cent of the activity of testosterone propionate and were determined from the minimal levels at which significant increases in seminal vesicle or levator ani muscle weights were obtained.

** Estrogenic compounds cause a small but significant increase in seminal vesicle weight but do not cause a corresponding increase in ventral prostate weight.

In addition to determining the minimal effective dose, the compounds were also studied at a uniform dose level. Since the 19-nor compounds were unesterified, testosterone, rather than testosterone propionate, was used as the standard. Figure 3 depicts the increases in weights of the levator ani muscle and of the seminal vesicles of rats treated with a total dose of 1 mg. of testosterone or 19-nortestosterone derivatives. Throughout the series of the 19-nor compounds, the myotrophic effects are relatively much more marked than are the androgenic effects. In fact, at this dose level the higher analogs do not show any androgenic effects. Furthermore, while 17-ethynyl-19-nortestosterone and its enol diacetate both increase seminal vesicle weight, such actions may be ascribed to their estrogenic effects, since estrogens as well as androgens cause an increase in seminal vesicle weight, but unlike androgens do not cause an increase in ventral prostate weight.

To determine oral activity, several of these compounds were administered by stomach tube daily over a period of 7 days. Solutions in corn oil were used and the results are presented in Table 3. 17-Ethyl-19-nortestosterone produced a myotrophic response at a lower dosage level than any other compound. As measured by the increase in seminal vesicle weight, the minimal dose of 17-ethyl-19-nortestosterone required to produce a

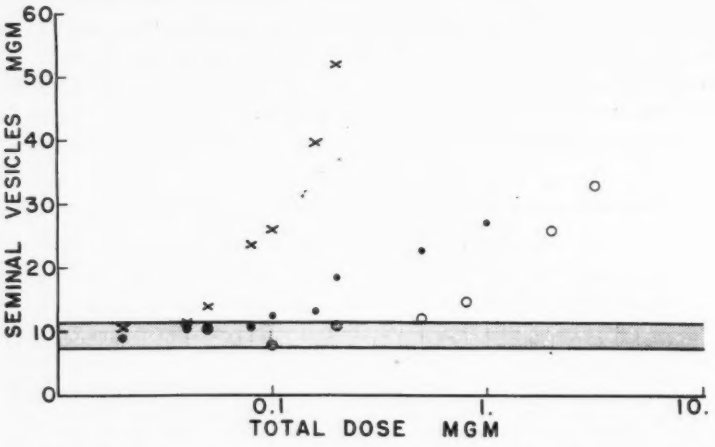

FIG. 2. The effect of several steroids on the seminal vesicle weight in castrate rats. Each point represents the average value obtained with 1 or more groups of 8 rats. The shaded area shows the range of average values obtained with 19 groups of controls. The crosses represent testosterone propionate; solid circles, testosterone; and open circles, 17-ethyl-19-nortestosterone.

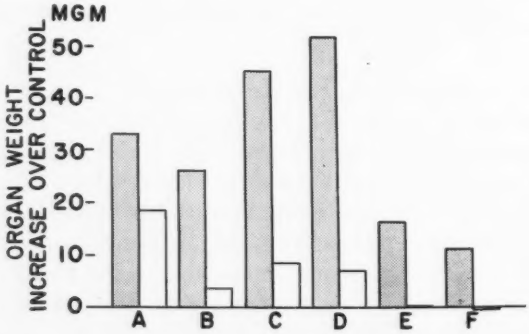

FIG. 3. Increases in the average weights of the levator ani muscles and the seminal vesicles of castrate rats after treatment with various steroids. In each case a total dose of 1 mg. was administered over a period of 7 days. The shaded bars represent the increase in levator ani muscle weight, while the open bars show the increase in the weight of the seminal vesicles. A = testosterone; B = 19-nortestosterone; C = 17-methyl-19-nortestosterone; D = 17-ethyl-19-nortestosterone; E = 17-propyl-19-nortestosterone; F = 17-butyl-19-nortestosterone.

significant increase was not much larger when administered orally than when given parenterally. However it is to be noted that with oral administration, at the doses used, none of the nor compounds produced a significant increase in the weight of the ventral prostate, whereas this gland was enlarged when methyl testosterone was given orally.

TABLE 3. ANDROGENIC AND MYOTROPHIC RESPONSE TO ORAL ADMINISTRATION IN CASTRATE MALE RATS. THE VALUES LISTED ARE THE DIFFERENCES IN AVERAGE WEIGHTS BETWEEN TREATED AND CONTROL GROUPS

Compound	Total dose	Seminal vesicles	Vental prostate	Levator ani
	mg.	mg.	mg.	mg.
19-nortestosterone	0.5	0.8	0.3	7.8
	1.0	−1.1	−2.7	−2.9
	2.0	−0.4	−2.4	8.7*
17-methyl-19-nortestosterone	0.2	0.6	−1.5	6.3
	0.5	0.9	1.0	8.9
	1.0	0.7	0.1	11.0
	2.0	4.0**	−1.0	20.0**
17-ethyl-19-nortestosterone	0.05	0.0	−0.9	−3.2
	0.1	2.0	1.4	−1.0
	0.2	0.4	1.4	12.7
	0.5	0.2	1.2	11.7
	1.0	1.4*	0.7*	15.1*
	2.0	2.2**	1.5	33.6**
Methyl testosterone	0.2	0.9	1.8	2.4
	0.5	0.4	1.3	−2.2
	1.0	0.9	4.0**	4.9
	2.0	3.8**	6.3**	15.6*
Testosterone propionate	0.2	0.2	0.8	0.9
	0.5	1.2**	0.4	3.9
	2.0	0.3	0.0	0.5
	5.0	0.7	2.2*	1.2

* $P = 0.05$.
** $P = 0.01$; unmarked values are not significantly different from controls.

DISCUSSION

The relatively low androgenic potency of the 19-nortestosterone compounds as reported by Hershberger *et al.* (1953) and Stafford *et al.* (1954), has been confirmed in our work. Our results show that the high myotrophic potency was retained in the 17-methyl and 17-ethyl compounds, but was lost when the 17 side chain was longer. Likewise, the substitution of a vinyl or ethynyl group for the ethyl group caused a loss in both androgenic and myotrophic potency. In metabolic studies (Saunders and Drill, 1956) it was found that the 17-methyl-19-nor compound inhibited the appetite of the rats. A study of the oral activity of these compounds showed that 17-ethyl-19-nortestosterone is approximately twice as effective as a myotrophic agent as either 17-methyl-19-nortestosterone or 19-nortestosterone itself. Likewise, the 17-ethyl-19-nortestosterone is at least twice as effec-

tive as methyl testosterone and more than 5 times as active as testosterone propionate when given intragastrically. In our studies, the latter compound was ineffective when given orally at the highest doses studied.

While it was shown (Table 2) that 19-nortestosterone, 17-methyl-19-nortestosterone and 17-ethyl-19-nortestosterone were approximately equal in their androgenic and myotrophic effects at minimal effective dose levels intramuscularly, as the dose was increased this relationship no longer held as strictly. It may be seen that at the 1 mg. dose level per rat (Fig. 3) the 17-ethyl compound produced twice as much increase in the weight of the levator ani muscle as did 19-nortestosterone. The 17-propyl and 17-butyl compounds not only showed much lower myotrophic potencies than those with the shorter side chains but, at the 1 mg. dose level, they were completely devoid of androgenic effects.

SUMMARY

The androgenic and myotrophic activities of testosterone have been compared to those of nortestosterone and to derivatives of nortestosterone in which the hydrogen on the 17 carbon atom has been replaced by methyl, ethyl, propyl, butyl, vinyl, or ethynyl radicals.

The 19-nor compounds uniformly exhibit relatively higher myotrophic potencies in comparison to their androgenic effects as measured by response of the levator ani muscle and seminal vesicles in the castrated rat.

With intramuscular administration, the minimal effective doses of 19-nortestosterone and the 17-methyl and 17-ethyl derivatives were similar. At a higher dose level of 1 mg., 17-ethyl-19-nortestosterone showed the greatest myotrophic effect.

On oral administration, 17-ethyl-19-nortestosterone was found to be more effective than 19-nortestosterone, 17-methyl-19-nortestosterone or methyl testosterone as measured by the levator ani response.

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STUDIES IN HISTOCHEMISTRY: EFFECTS OF STRESS CONDITIONS, ACTH, CORTISONE, DESOXYCORTICOSTERONE AND HYPOPHYSECTOMY ON THE QUANTITATIVE HISTOLOGICAL DISTRIBUTION OF PHENOLSULFATASE AND ACETYLCHOLINESTERASE IN THE RAT ADRENAL[1,2]

DAVID GLICK AND HELEN R. STECKLEIN

Histochemical Laboratory, Department of Physiological Chemistry, The Medical School, University of Minnesota, Minneapolis, Minnesota

THE quantitative histological distribution of phenolsulfatase (Glick, Stecklein and Malmstrom, 1955) and acetylcholinesterase (Antopol and Glick, 1940) in the adrenal gland of various species was reported earlier. The greatest phenolsulfatase activity was found in the fascicular and reticular zones of the rat, rabbit and hog adrenal and in the fascicular zone of the dog adrenal, but negligible activity was demonstrated in all regions of the cow and monkey gland. Considerable acetylcholinesterase activity was observed in the medulla of bovine, pig and rabbit adrenals but little activity was found in any cortical zone in any of the species.

The effects of stress conditions, hormonal treatments and hypophysectomy on the adrenal have been studied more extensively in the rat than in other laboratory animals, and consequently the rat adrenal was used in the present investigation to permit comparisons. For the phenolsulfatase work the rats were subjected to chilling, turpentine abscesses, hypophysectomy and injections of ACTH, cortisone and desoxycorticosterone. The same kinds of treatments were used for the acetylcholinesterase work except that the chilling experiments were omitted after it became apparent that stress conditions produced no appreciable change.

EXPERIMENTAL

Male albino rats (Sprague-Dawley) weighing 350–500 gm. were used for all of the experiments. For the hypophysectomy experiments unoperated, sham-operated and hypophysectomized rats were obtained from the Hormone Assay Laboratories, Inc., Chicago. For the other work the rats were obtained from the Holtzman Co., Madison, Wis.

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The animals were housed in a room of constant temperature (78 ± 1° F) and relative humidity (30–50%). Instantaneous death was inflicted by a single hammer blow on the head, and always at the same time of day (10 A.M.). The adrenals were quickly removed, frozen with solid carbon dioxide and stored at −20° C. The procedures for obtaining the microtome sections of fresh frozen adrenal tissue for chemical analysis and histological examination were the same as previously employed by Malmstrom and Glick (1952), Bahn and Glick (1954), and Glick *et al.* (1955). For each sample of one, or more than one, tissue sections used for chemical analysis, the previous adjacent section was taken for histological identification after fixing in Carnoy's solution and staining with toluidine blue, and the following adjacent section was used for protein-nitrogen determination.

The determination of phenolsulfatase activity followed the steps outlined by Glick, Stecklein and Malmstrom (1955) which represents a modification of the method of Malmstrom and Glick (1952). Protein-nitrogen was measured by the bromsulfalein procedure described by Nayyar and Glick (1954). The cholinesterase determinations were based on the titrimetric method of Glick (1938a) with modifications in certain details to give the following procedure:

Procedure for cholinesterase determination

1. Pipet 9 μl. of 30% glycerol into each reaction tube.
2. Insert tissue sample into the liquid by means of a glass needle. Allow to extract for 1 hr. at room temperature.
3. Add 32 μl. of 0.022 M acetylcholine chloride or bromide made up in 0.1 M veronal buffer, pH 8.0. (Prepare buffer by combining 7.15 ml. of 0.1 M sodium diethylbarbiturate and 2.85 ml. of 0.1 M HCl.) Dissolve substrate in buffer just before use. (Purify commercial substrate by dissolving 1 gm. in 13 ml. of absolute alcohol, add 22.5 ml. of absolute ether, cool in ice in stoppered vessel, quickly filter off solid on a Buchner funnel and store in a desiccator over P_2O_5.)
4. Include non-substrate blanks in which no buffered substrate is added to tissue in glycerol solution. For the non-enzyme blank place about 0.5 ml. of buffered substrate in a vessel and stopper it.
5. Digest samples from step 3, and blanks from step 4 simultaneously for 4 hr. at 37° C.
6. At the end of the digestion add 50 μl. of eserine-indicator solution (10 ml. of 0.1% eserine sulfate plus 1.5 ml. of 0.04% bromothymol blue) to each tube containing tissue, including non-substrate blanks, and mix.
7. To each non-substrate blank add 32 μl. of the buffered substrate which had been digested (non-enzyme blank), and mix.
8. Titrate all tubes including those containing the combined blank with 0.05 N HCl to pH 6.2 using a color standard consisting of M/15 phosphate buffer, pH 6.2, containing indicator (10 ml. phosphate buffer plus 0.75 ml. 0.04% bromothymol blue).
9. Calculate activities as μl. of acid used in titration of the combined blank minus μl. of acid used in titration of the enzyme runs.

Estimates of the enzyme activity that might be contributed by the presence of blood in the tissue revealed that for both enzymes the contribution was negligible.

The data obtained were represented in composite curves giving chemical values as a function of distance through the adrenal. These curves were constructed in a manner similar to that described by Bahn and Glick (1954).

RESULTS

Phenolsulfatase

The effect of the ACTH and DCA injections on either the phenolsulfatase distribution or activity in the rat adrenal was found to be negligible

(Fig. 1). This applied whether the activity was based on unit volume of fresh tissue (per section) or on the protein-nitrogen content. When cortisone treatment was given, a slight tendency to elevated activities per tissue section was observed but this was eliminated when the activities were related to protein-nitrogen because the latter also tended to be high in the inner portions of the gland (Fig. 2).

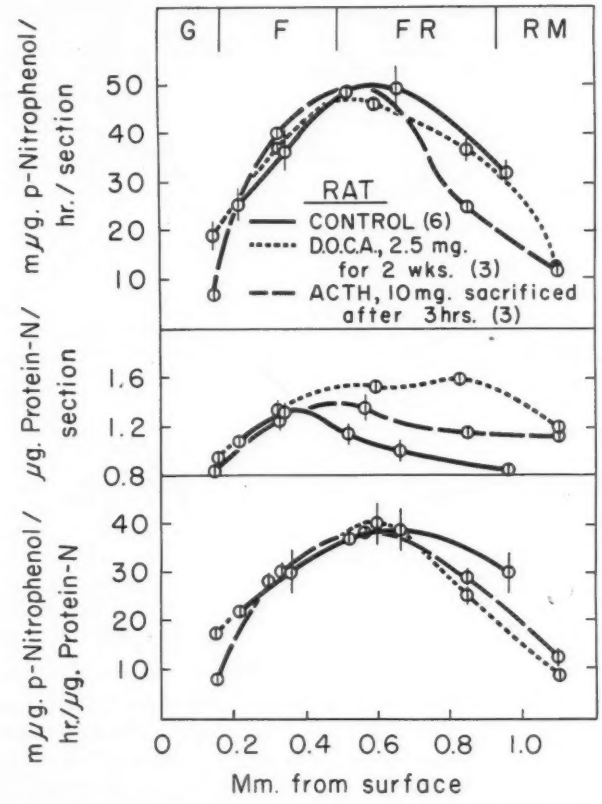

FIG. 1. Distribution of phenolsulfatase activity and protein-nitrogen in the adrenal glands of male albino rats with and without daily subcutaneous injections of 7 mg. desoxycorticosterone acetate in sesame oil/kg. for 2 weeks, and with and without single subcutaneous injections of 25 mg. ACTH/kg. Sections 2 mm. diameter, 16 μ thick (0.05 μl. vol.). In this, and in the subsequent figures, the following apply: in curves of activity per tissue section and of protein-nitrogen per section, the points represent means and the vertical lines through them the standard error of the means. In curves of activity per unit protein-nitrogen, the points and vertical lines are ratios of the corresponding values for activities per section to protein-nitrogen per section. Histological zones are indicated by G (glomerulosa), F (fasciculata), R (reticularis), M (medulla). When sections are mixtures of two zones both letters are used. Numbers in parenthesis after the designation of treatments denote the number of adrenals analyzed to obtain the composite curve.

A condition of stress imposed by the development of turpentine abscesses tended to reduce the activities per section, but also in this instance the activities per unit protein-nitrogen were not appreciably changed (Fig. 2). Furthermore, chilling at 2° C for periods of 20, 60, and 120 minutes failed to affect the activities when they were referred either to tissue volume or to protein-nitrogen (Fig. 2).

Hypophysectomy caused a marked decline in activities in the fascicular and reticular zones without changing the general distribution profile, *i.e.*,

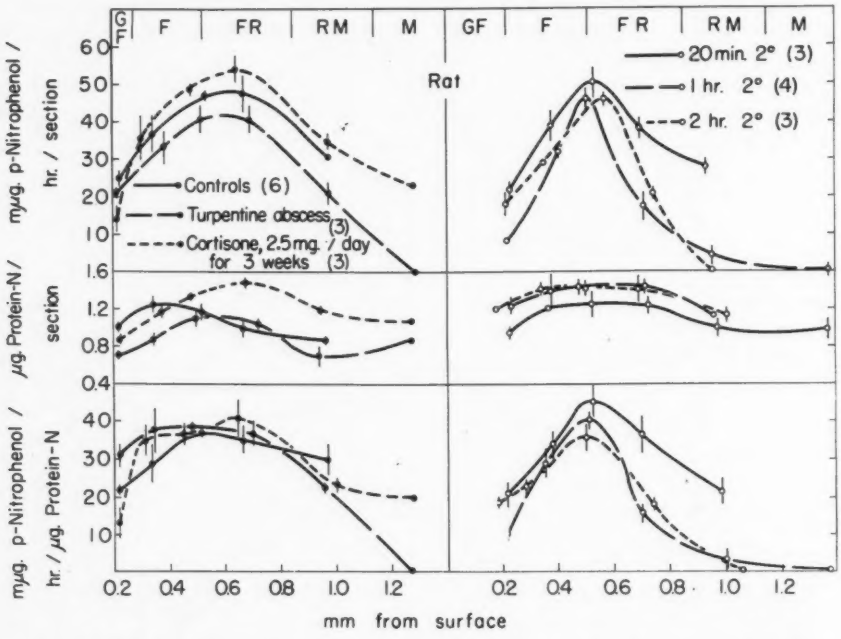

FIG. 2. Effects of turpentine abscesses (one week after single subcutaneous injections of 0.5 ml. of turpentine per rat), repeated subcutaneous injections of 7 mg. cortisone acetate/kg., and exposure to cold for various periods, on the distribution of phenol-sulfatase activity and protein-nitrogen in adrenal glands of male ablino rats. Sections 2 mm. diameter, 16 μ thick (0.05 μl. vol.).

the peak of activity still occurred in the inner fasciculata with a falling off on both sides (Fig. 3). The decrease in activities by the sixth day after hypophysectomy was almost as great as that observed after 30 days.

Acetylcholinesterase

In Figure 4 it may be seen that the enzyme activity is concentrated in the medulla, and that it falls off precipitously through the reticularis to the fasciculata and glomerulosa in which activities were not demonstrable. The influence of treatments with cortisone, DCA in sesame oil, sesame oil

alone, and the effects of turpentine abscesses, all were practically negligible. However a single injection of ACTH reduced the medullary activity per section but this effect largely disappeared when the activity was based on protein-nitrogen.

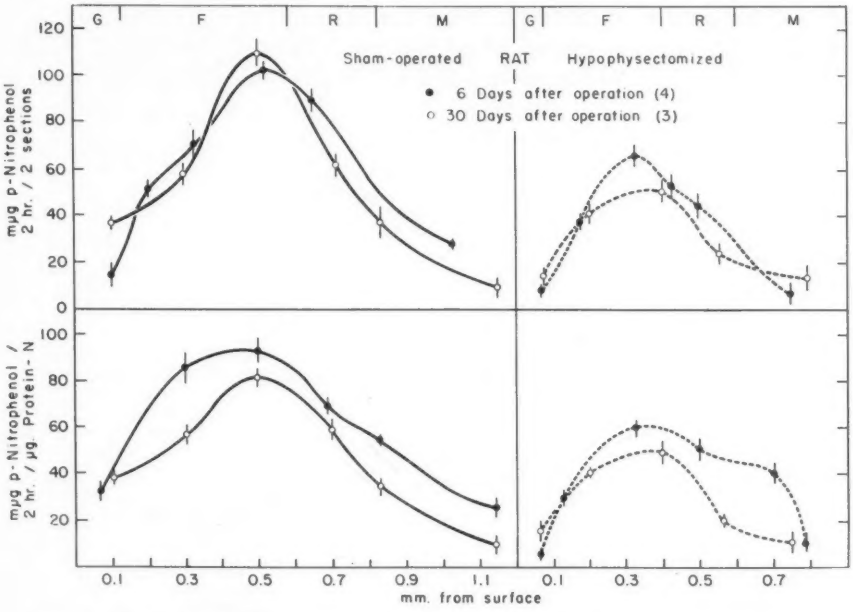

FIG. 3. Effect of hypophysectomy on the phenolsulfatase activity in adrenal glands of male albino rats. Sections 1.5 mm. diameter, 16 μ thick (0.028 μl. vol.).

Twelve days after hypophysectomy a rise in medullary activity per section seemed to have occurred, but this influence was also essentially eliminated when the activity was referred to the protein-nitrogen content (Fig. 5). In the adrenals of the rats used for the hypophysectomy and sham-operation some activity was observed in the inner fasciculata but this fell rapidly to zero in the middle and outer regions of this zone.

DISCUSSION

From the data presented it is clear that the treatment of rats with doses of ACTH, DCA, and cortisone which are known to produce physiological effects had little influence on the histological distribution or magnitude of the phenolsulfatase activity. Furthermore the stress conditions of turpentine abscess or cold also had practically no effect. This refractory state is all the more interesting in the light of the fact that the adrenal contains one of the very highest concentrations of this enzyme in the animal body, Rutenburg *et al.* (1952), Dodgson *et al.* (1953). This situation is parallel

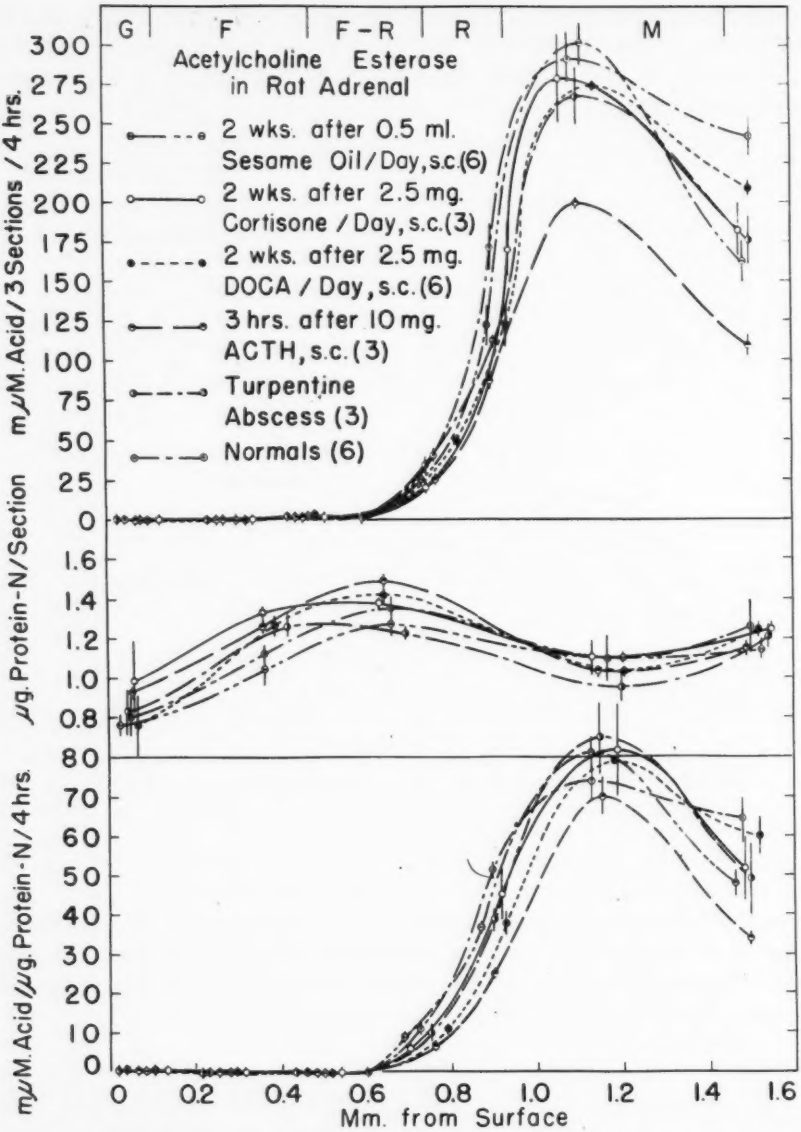
G
F
F-R
R
M
Acetylcholine Esterase in Rat Adrenal
2 wks. after 0.5 ml. Sesame Oil/Day, s.c.(6)
2 wks. after 2.5 mg. Cortisone / Day, s.c.(3)
2 wks. after 2.5 mg. DOCA / Day, s.c.(6)
3 hrs. after 10 mg. ACTH, s.c.(3)
Turpentine Abscess (3)
Normals (6)
mμM. Acid / 3 Sections / 4 hrs.
μg. Protein-N / Section
mμM. Acid / μg. Protein-N / 4 hrs.
Mm. from Surface

FIG. 4. Distribution of acetylcholinesterase activity and protein-nitrogen in adrenal glands of male albino rats with and without turpentine abscesses (2.5 weeks after single subcutaneous injections of 0.5 ml. of turpentine per rat), single subcutaneous injections of 25 mg. ACTH/kg., repeated subcutaneous injections of 7 mg. cortisone acetate/kg., repeated subcutaneous injections of 7 mg. DOCA in sesame oil/kg., and repeated subcutaneous injections of sesame oil alone. Sections 2.0 mm. diameter, 16 μ thick (0.05 μl. vol.).

to that reported for β-glucuronidase by Nayyar and Glick (1955) in which similar hormonal treatments and also stress conditions in monkeys effected no significant change (the distribution and magnitude of the β-glucuronidase activities in the adrenals of the rat and monkey were similar). Both of these enzymes most likely function exclusively in a hydrolytic manner (a system for synthesis of sulfate or glucuronate conjugation by rat adrenal was tested by Glick and Stecklein (1955) and no synthetic action could be demonstrated) and both enzymes are capable of the hydrolysis of hormone conjugates.

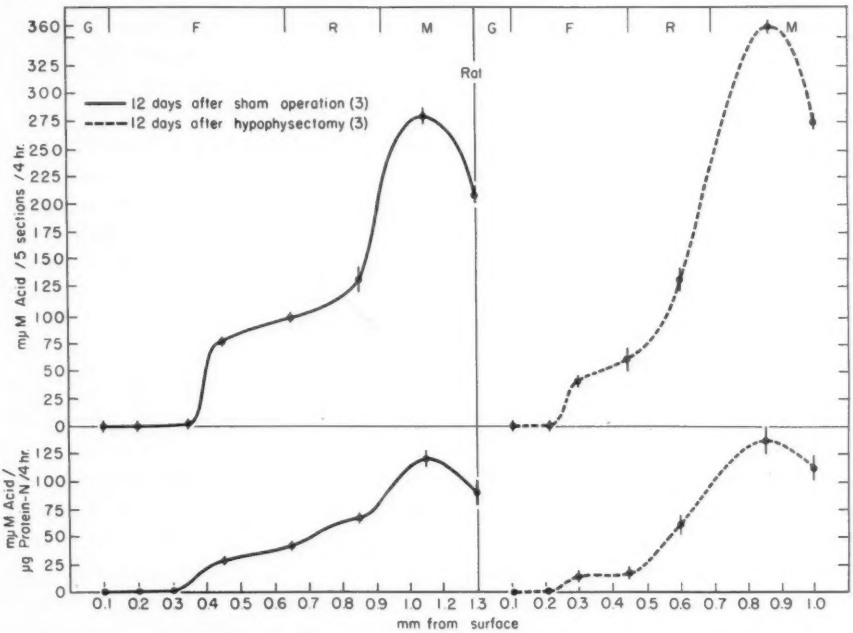

FIG. 5. Effect of hypophysectomy on acetylcholinesterase activity in adrenal glands of male albino rats. Sections 1.5 mm. diameter, 16 μ thick (0.028 μl. vol.).

The parellelism between phenolsulfatase and β-glucuronidase in the adrenal breaks down in a comparison of their responses to hypophysectomy of the rat. Whereas the former enzyme underwent a marked fall in concentration in the fascicular and reticular zones, the latter had been shown by Nayyar and Glick (1955) to rise in all zones, particularly, however, in the fasciculata and reticularis.

To arrive at a rationale for the increased concentration of β-glucuronidase in the adrenal following hypophysectomy, Nayyar and Glick (1955) suggested the possibility that the increased activity per unit of tissue might be an attempt to liberate more free hormone from the blood pool

of conjugate to compensate in some degree for the decreased hormone synthesis in the atrophic adrenal. Should this prove to be a valid explanation, it certainly could not apply to the change in phenolsulfatase activity which goes in the opposite direction. We are left with a situation now more puzzling than ever. A point which may be related, in that sulfatase is a type of esterase, is that an inverse relationship has been reported between the changes in β-glucuronidase and esterase (methyl butyrase) activities of mouse and human tissues following treatments with steroidal hormones (Cohen, 1951).

As in the case of phenolsulfatase and β-glucuronidase, no demonstrable changes in acetylcholinesterase activities were found after the stress and hormonal treatments. Glick (1938b) had shown that acetylcholinesterase activities in the gastric mucosa of the hog were not influenced by the atropine inhibition of secretion or by the acetylcholine or eserine stimulation; furthermore, the activities were essentially the same in the fasted and fed state. It would appear that the enzymes in question exist in concentrations which are remarkably constant and independent of physiological and even certain pharmacological influences. While the significance of this is not clear, it may be that a relatively fixed excess of enzyme is provided in these cases so that their function can continue essentially unchanged in spite of a considerable range of physiological variation.

The pronounced changes in enzyme concentration following hypophysectomy which had been observed for phenolsulfatase and β-glucuronidase did not occur for acetylcholinesterase. Again, the reason for this is not apparent.

Although injections of ACTH had little effect on the acetylcholinesterase activities in the experiments presented, preparations of this hormone activate the enzyme *in vitro* according to Mian (1951). Greig and Carter (1954) found that the enzyme decreased in activity in rat kidney following adrenalectomy, but that a reactivation *in vitro* could be effected by adrenal cortex extract, cortisone, or ouabain; DCA had no influence. The kidney enzyme from untreated rats was either unaffected or inhibited by these same preparations.

The greater localization of phenolsulfatase in the fascicular and reticular zones, as reported in this paper, may be compared with the staining results of Rutenburg *et al.* (1952) who observed most intense coloring in the fasciculata, although all cortical zones were stained. The localization of acetylcholinesterase primarily to the medulla, as revealed by the quatitative method, is in accord with the results by staining of Gomori, 1948, Koelle and Friedenwald, 1949, and Koelle, 1951.

SUMMARY

Treatment of male albino rats with ACTH, DCA, or cortisone, and subjecting them to the stress conditions of turpentine abscess or cold,

failed to cause demonstrable changes in the concentration or histological distribution of phenolsulfatase activity based on the protein-nitrogen content. Six days after hypophysectomy a marked decline in the activities in the fascicular and reticular zones was found, and this effect was almost as great as that observed after thirty days. The enzyme was localized primarily in the inner fasciculata and outer reticularis.

Similar studies on acetylcholinesterase revealed that the same hormone treatments and turpentine abscess also were without apparent effect. Hypophysectomy had minor influences on the magnitude of the activity or distribution of the enzyme. The enzyme was localized primarily in the medulla.

Acknowledgment

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STUDIES IN HISTOCHEMISTRY: EFFECTS OF ACTH, CORTISONE, AND DESOXYCORTICOSTERONE ON THE QUANTITATIVE HISTOLOGICAL DISTRIBUTION OF POTASSIUM IN THE MONKEY AND GUINEA PIG ADRENAL[1,2]

RICHARD H. SWIGART,[3] HELEN R. STECKLEIN AND DAVID GLICK

Histochemical Laboratory, Department of Physiological Chemistry, The Medical School, University of Minnesota, Minneapolis, Minnesota

INTRODUCTION

IT HAS been the purpose of the current series of studies on the adrenal gland to map out the quantitative histological distribution of substances and activities of functional importance, and to follow the effects of altered physiological states. Among the cations present in this tissue, potassium is of particular interest for its prominent role in cell function. The idea was entertained that certain responses of the adrenal gland to changes in the physiological state of the organism might be associated with changes in potassium ion concentrations in the tissue.

Earlier Glick *et al.* (1955) found that the potassium concentration was relatively constant throughout all of the histological zones of the monkey and guinea pig adrenal with the exception of a small, but consistent, increase on a fat-free dry weight basis, in the border area between the glomerulosa and fasciculata. This work has now been extended to include a study of the effects of ACTH, cortisone and DCA on the adrenals of the same species.

EXPERIMENTAL

The *M. rhesus* and *M. cynomolgus* monkeys used were those which had been employed by Bahn and Glick (1954) for a study of ascorbic acid in the adrenal. They were housed, fed, and treated as described previously. Female guinea pigs weighing 500–900 gm. and male albino rats (Sprague-Dawley) weighing 350–500 gm. were used.

The monkeys were killed by shooting in the head as described by Glick *et al.* (1954), and the guinea pigs were killed by a single hammer blow on the head. All animals were

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[3] Present address, Department of Anatomy, University of Louisville, Ky.

killed at the same time of day (10 A.M.).

The adrenals were quickly removed, frozen with solid carbon dioxide and stored at −20° C. Microtome sections of fresh frozen tissue were obtained for chemical analysis and histological examination by the procedures described by Bahn and Glick (1954) and Glick *et al.* (1955). For each sample of consecutive tissue sections taken for chemical analysis, the previous adjacent section was taken for histological identification. The latter was made after fixing in Carnoy's solution and staining with toluidine blue.

The procedures used for total dry weight, fat-free dry weight, and potassium determination by flame photometry were those described by Glick *et al.* (1955).

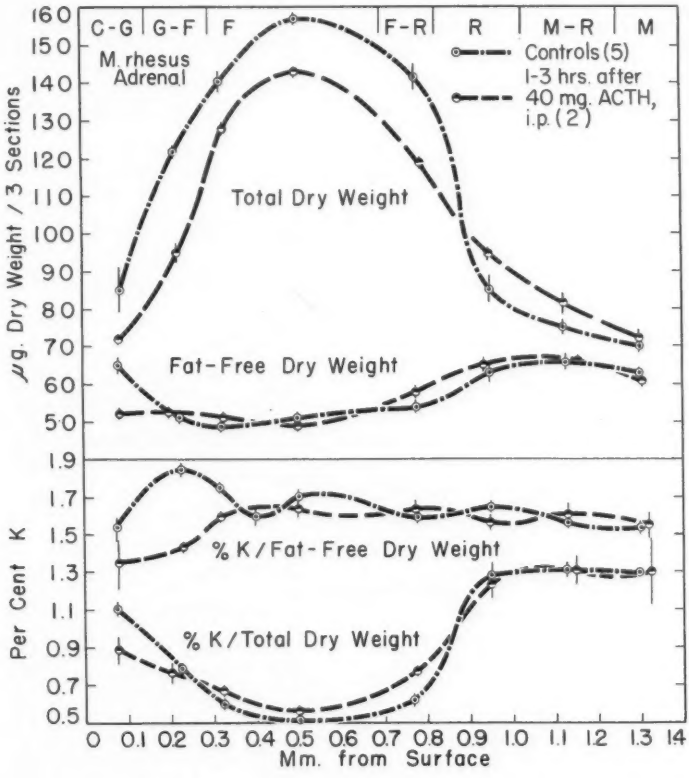

FIG. 1. Distribution of total dry material, fat-free dry material, and potassium in adrenal glands from *M. rhesus* monkeys with and without single intraperitoneal injections of 40 mg. of ACTH (15 mg./kg.). Each tissue section was 3 mm. diameter, 16 μ thick, 0.11 μl. vol. In all of the figures the following apply: the points represent means, vertical lines through points, the standard error of means. Histological zones are indicated on the top of each graph by C, G, F, R and M denoting capsule, glomerulosa, fasciculata, reticularis and medulla, respectively. Sections which are mixtures of two zones are indicated by the use of both letters. Numbers in parenthesis after the names of the species or treatment denote the number of adrenals analyzed to obtain the composite curve.

RESULTS

It is apparent from Fig. 1 that within 3 hours after the intraperitoneal injection of 40 mg. of ACTH into *M. rhesus* monkeys little effect on the potassium concentration was obtained compared to the untreated controls, with the exception of a tendency to lower values on a fat-free dry weight basis in the glomerular and bordering fascicular zones. As might be expected, the fat-free dry weight was unchanged by the treatment but the total dry weight was reduced in the outer and middle regions of the cortex, primarily due to the loss of cholesterol ester from the fasciculata, Glick and Ochs (1955). The latter effect was exaggerated by the increased dose (100 mg.) of ACTH given the *M. cynomolgus* monkeys (Fig. 2), but

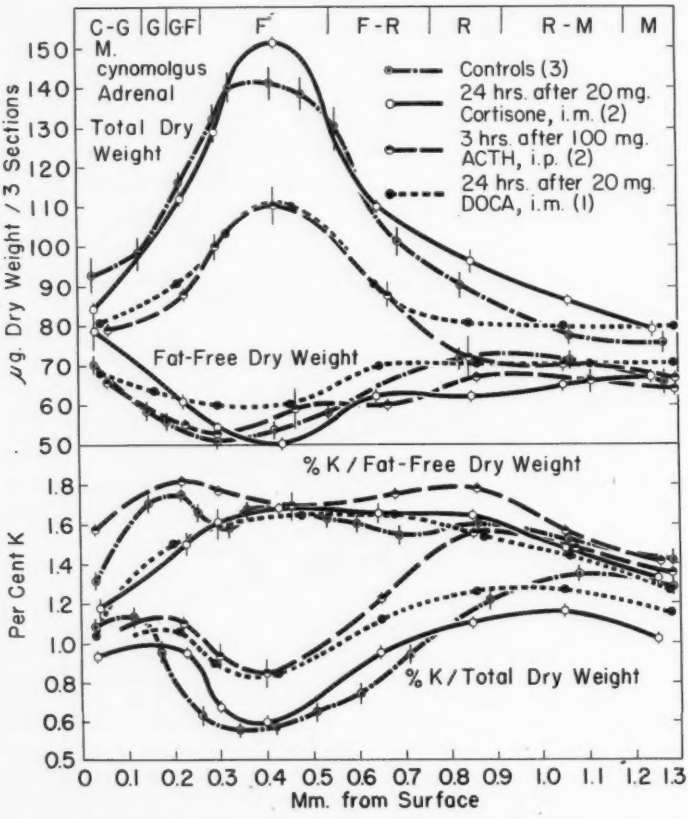

FIG. 2. Distribution of total dry material, fat-free dry material, and potassium in adrenal glands from *M. cynomolgus* monkeys after single intraperitoneal injections of 100 mg. of ACTH (30 mg./kg.), or single intramuscular injections of 20 mg. of cortisone acetate (10 mg./kg.), or single intramuscular injections of 20 mg. of DCA (10 mg./kg.). Each section was 3 mm. diameter, 16 μ thick, 0.11 μl. vol.

the tendency toward lower glomerular potassium values which was noted with the smaller dose in the *M. rhesus* monkeys was absent. Results obtained with the guinea pig adrenal following the subcutaneous injection of 10 mg. of ACTH are given in Fig. 3, and higher values for the potassium concentration in the reticular zone, compared to the controls, may be noted.

The cortisone treatment of the *M. cynomolgus* monkey (Fig. 2) appears to have had no effect on the dry weight values, but the tendency toward lower glomerular potassium values per unit fat-free dry weight, which were observed after injection of the lower dose of ACTH in the *M. rhesus* monkeys, occurred in these cases. Higher concentrations of potassium in all zones of the guinea pig adrenal (Fig. 3) were found but the profile of the distribution curve was essentially unchanged from that of the control.

The DCA treatment of the monkey (Fig. 2) produced the same effect on the dry weight values as the ACTH injection; but its effect on the potas-

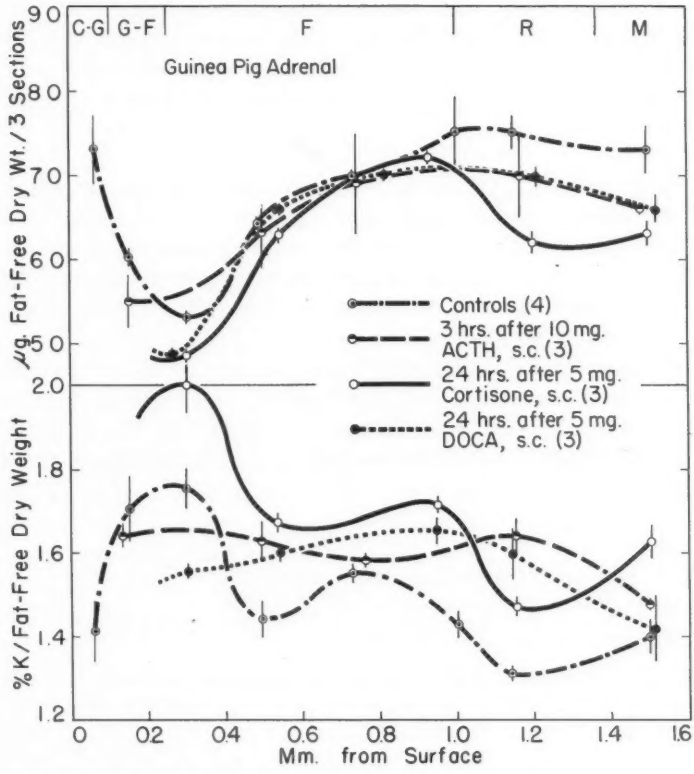

FIG. 3. Distribution of fat-free dry material and potassium in adrenal glands from guinea pigs with and without single subcutaneous injections of 10 mg. of ACTH (15 mg./kg.), or 5 mg. of cortisone (7.5 mg./kg.), or 5 mg. of DCA (7.5 mg./kg.). Each section was 3 mm. diameter, 16 μ thick, 0.11 μl. vol.

sium per unit fat-free dry weight was the same as that observed following the cortisone injection. In the guinea pig adrenal (Fig. 3) the potassium values following DCA administration were similar to those obtained after ACTH.

DISCUSSION

From the data obtained, it is doubtful whether any effect on the potassium content per unit fat-free weight in any adrenal zone can be ascribed to the single injections of the hormones used, although these injections are known to affect the functional state of the organ. The differences noted, compared to the controls, did not seem to follow a pattern consistent with any clear interpretation. If the differences seen are indeed significant, further evidence would be required to establish it. Changes that may have occurred at the cell level might not influence the overall concentrations observed in microtome sections.

The loss of total lipid from the cortex of the monkey adrenal following single injections of ACTH or DCA, and the lack of appreciable effect from a single injection of cortisone, parallel the results obtained by Glick and Ochs (1955) for depletion of cholesterol ester after similar treatments.

SUMMARY

The quantitative histological distribution of potassium in monkey and guinea pig adrenals was studied following single injections of ACTH, cortisone, and DCA in doses sufficient to affect adrenal function. No significant effects on the per cent potassium per unit fat-free dry weight could be ascribed to any of these treatments although certain variations were noted.

Depletion of total lipid material from the monkey adrenal, principally from the fascicular zone, after injections of ACTH and DCA, and the absence of this effect after cortisone, were similar to the changes which had been previously observed in the cholesterol ester concentration following similar treatments.

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PHOSPHORUS METABOLISM OF THE ADRENAL GLAND: EFFECT OF COLD STRESS ON THE LABELING OF PHOSPHOLIPID AND RNA NUCLEOTIDES WITH RADIOACTIVE INORGANIC PHOSPHATE[1]

DORIS NICHOLLS,[2] F. C. HEAGY[3] AND R. J. ROSSITER
Department of Biochemistry, University of Western Ontario, London Canada

PREVIOUSLY it was reported that exposure of rats to a cold environment ($3 \pm 1°$ C) for both short periods (2, 3, 6 hours) and somewhat longer ones (2, 4, 8, 16 days) caused an increase in the incorporation of radioactive phosphorus (P^{32}) into the inorganic phosphate, 20-minute hydrolyzable phosphorus, and total acid-soluble phosphorus of the adrenal gland, without concomitant change in phosphorus concentration. Evidence was presented for the view that this could be explained by an increase in the rate at which inorganic P^{32} passes from the extracellular to the cellular portion of the gland (Nicholls and Rossiter, 1955a). Furthermore, there is good evidence that the early (2 or 3 hours in the cold) response in the adrenal phosphorus metabolism is caused by the release of endogenous ACTH from the pituitary, and that the later response (8 days in the cold) is, in part at least, associated with an increase in the activity of the thyroid gland (Nicholls and Rossiter, 1955b).

It is well established that removal of the pituitary gland causes a decrease in the incorporation of inorganic P^{32} into the total acid-soluble phosphorus fraction (Reiss and Halkerston, 1950), inorganic phosphate (Gemzell and Samuels, 1950; Riedel, Logan, DeLuca and Rossiter, 1954a), lipid phosphorus (Riedel, Logan and Rossiter, 1954b) and ribonucleic acid (RNA) phosphorus (Logan, Heagy and Rossiter, 1955) of the adrenal gland. In each instance, the changes brought about by hypophysectomy are reversed rapidly by a single injection of ACTH.

For the acid-soluble phosphorus compounds, the effect of a short exposure of the animal to a cold environment is similar to that produced by the administration of ACTH to a hypophysectomized animal. If the changes in adrenal phosphate metabolism that follow a short period of cold stress are produced by the liberation of ACTH from the pituitary, as suggested by Nicholls and Rossiter (1955b), it follows that such endog-

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[3] Senior Medical Fellow, National Research Council of Canada, 1952–55.

enous ACTH should produce the changes in the labeling of phospholipid and RNA reported by Riedel *et al.* (1954b) and Logan *et al.* (1955) for exogenous ACTH. The experiments described in the present paper were designed to test this hypothesis.

METHODS

Experimental Procedure

Male rats (85–160 gm.) of the Sprague-Dawley strain, maintained at an environmental temperature of $22 \pm 1°$ C, were injected intraperitoneally with 200 μc. P^{32} as inorganic phosphate.[4] The animals either were maintained at the same environmental temperature of 22° C, or were transferred for the next 16 hours to a cold room maintained at $3 \pm 1°$ C. At the end of this time blood samples were obtained and the adrenal glands removed as described previously (Nicholls and Rossiter, 1955a). During the experiment all animals were allowed free access to food (Master Fox Cubes, Toronto Elevators, Ltd.) and water.

Analytical and Counting Methods

The adrenals from nine rats were pooled to provide sufficient tissue for the isolation of the RNA nucleotides. The adrenals were frozen rapidly by plunging into liquid nitrogen, weighed in the frozen state, and then homogenized in ice-cold 10% trichloroacetic acid using a homogenizer of the Potter-Elvehjem type. The concentration and the specific activity of the inorganic P of the trichloroacetic acid extracts of a pooled sample of plasma and the pooled adrenals were determined by the method of Ernster, Zetterström, and Lindberg (1950), as described by Nicholls and Rossiter (1955a). The tissue residue was washed and the lipids extracted for the determination of the concentration and specific activity of the lipid P, as described by Riedel *et al.* (1954, 1954b). The nucleic acids then were extracted and the specific activity of the individual RNA nucleotides determined after separation by ionophoresis on filter paper by the method of Davidson and Smellie (1952). Details of the method may be found in the paper by Logan *et al.* (1955).

In experiments in which the concentration of nucleic acid P was determined, the adrenals from two animals were pooled. The tissue residue remaining after extraction of the lipids was hydrolyzed for 18 hours at 37° C in 1.0N KOH. The RNA was separated from the desoxyribonucleic acid (DNA) by acid precipitation of DNA and insoluble protein according to the method of Schmidt and Thannhauser (1945). The RNA phosphorus was estimated as the difference between the total P and the inorganic P in the supernatant. No correction was made for non-nucleotide organic P (Logan, Mannell and Rossiter, 1952). The DNA phosphorus was estimated as the difference between the total P in the hydrolysate and that in the supernatant.

Radioactivity was measured with an M-6 liquid counter (20th Century Electronics). Counting rates were corrected for background and decay. Specific activities (expressed as counts/min./μg. P) were adjusted to a standard injection of 4.3×10^7 counts per minute per 200 gm. body weight.

RESULTS

Plasma Inorganic Phosphate

Table 1 shows that the specific activity of the inorganic P of the plasma, 16 hours after the injection of P^{32}, was not changed significantly by exposure of the animals to cold for the 16 hours of the experiment. This find-

[4] Obtained from Commercial Products Division, Atomic Energy of Canada, Limited.

ing is consistent with previous experiments, where the specific activity of the plasma inorganic P was determined either 2 or 3 hours after P^{32} administration in rats exposed to cold for 2, 3, 6 or 24 hours (Nicholls and Rossiter, 1955a).

Table 1 also shows that after the animals had been in the cold for 16 hours there was a statistically significant, though small, increase in the concentration of inorganic P in the plasma. A similar increase was found in the previous study for animals exposed to the cold for 6 and 24 hours (Nicholls and Rossiter, 1955a).

Adrenal Inorganic Phosphate

There was a significant increase in both the specific activity of the adrenal inorganic P and the specific activity of the adrenal inorganic P relative to that of the inorganic P of the plasma in animals exposed to the

TABLE 1. EFFECT OF EXPOSURE TO THE COLD (3 ± 1° C) FOR 16 HOURS ON THE CONCENTRATION AND SPECIFIC ACTIVITY OF THE INORGANIC PHOSPHORUS OF THE PLASMA AND OF THE ADRENAL GLAND OF THE RAT

Group	No. Determinations	Plasma inorganic P		Adrenal inorganic P		
		Concentration (mg./100 ml.)	Specific activity* (cts./min./μg.P)	Concentration (mg./100 gm.)	Specific activity (cts./min./μg.P)	Specific activity relative to plasma inorganic P × 10^2
Room temperature	15	8.99 ± 0.15†	50.8 ± 2.0	14.7 ± 0.56	52.8 ± 2.5	104 ± 2.5
16 hours cold	7	10.8 ± 0.18	52.3 ± 2.1	14.4 ± 0.82	68.0 ± 3.3	130 ± 3.9
P[1]		<0.001	>0.6	>0.7	<0.001	<0.001

* Injected dose, 4.3×10^7 cts./min. for a 200 gm. rat.
† Mean ± standard error.
[1] P value for animals in the cold compared with control rats at room temperature.

cold for 16 hours (Table 1). A similar increase was reported for the relative specific activity of the adrenal inorganic P 2 or 3 hours after P^{32} administration in rats exposed to cold for 2 or 3 hours, respectively (Nicholls and Rossiter, 1955a). In other experiments, the increase was observed 30 minutes after P^{32} administration in rats exposed to cold for as little as 30 minutes.

No significant change in the concentration of inorganic P in the adrenal was observed, either in the present experiments (Table 1), or in previous experiments in which the animals were exposed to cold for 2, 6 or 24 hours (Nicholls and Rossiter, 1955a).

The increase in the specific activity of the inorganic P of the adrenal may represent an increase in the specific activity of the cellular inorganic P. It should be borne in mind that the specific activity of the whole gland,

a short time after the injection of P^{32}, does not necessarily give an estimate of the specific activity of the cellular inorganic P, since there may be unequal penetration of the isotope into the extracellular and cellular compartments of the gland. The measured inorganic P of the whole gland is partly extracellular (plasma and interstitial fluid) and partly cellular. The difficulties of determining the specific activity of the cellular inorganic P are well known. However, if the specific activity of the cellular inorganic P is calculated by the method of Hevesy (1948), making the assumptions previously described (Nicholls and Rossiter, 1955a), it is found to be increased significantly and consistently in the rats exposed to the cold.

Figure 1 shows the calculated specific activity of the cellular inorganic P of the adrenal gland at different times after P^{32} administration for ani-

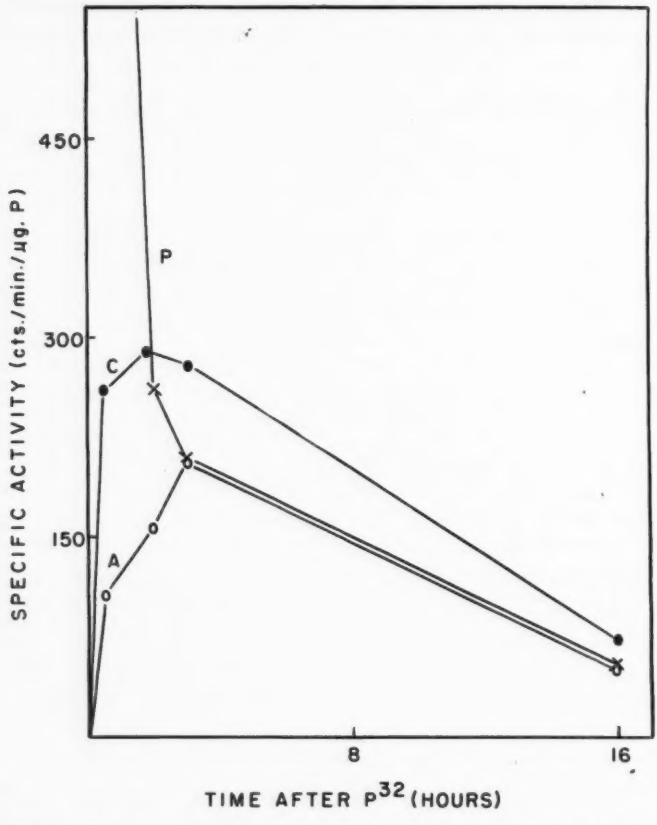

FIG. 1. Effect of exposure to the cold (3 ± 1° C) on the specific activity of the inorganic phosphate of the plasma and the intracellular fluid of the adrenal gland of the rat. P(x-x-x), plasma inorganic P; A(o-o-o), adrenal cellular inorganic P for rats at 22°; C(●-●-●), adrenal cellular inorganic P for rats at 3°. Each point represents the mean of 9 or more animals.

mals either maintained at room temperature, or exposed to the cold at the same time as the isotope injection. The figure also shows the specific activity of the inorganic P of the plasma, which was not significantly different for the two groups.

As found previously by Riedel and Rossiter (1954), the specific activity of the cellular inorganic P of the adrenal gland of the rats maintained at room temperature reached that of the inorganic P of the plasma approximately 3 hours after the P^{32} administration and thereafter the two specific activities declined together. For the animals maintained in the cold, however, the specific activity of the cellular inorganic P of the adrenal gland reached that of the inorganic P of the plasma much more rapidly (less than 2 hours). During the first 16 hours after the P^{32} injection the specific activity of the cellular inorganic P remained greater than that of the inorganic P of the plasma in the cold-exposed animals.

TABLE 2. EFFECT OF EXPOSURE TO THE COLD ($3 \pm 1°$ C) FOR 16 HOURS ON THE SPECIFIC ACTIVITY OF THE LIPID PHOSPHORUS OF THE ADRENAL GLAND OF THE RAT

Group	No. determinations	Specific activity* (cts./min./μg. P)	Specific activity relative to plasma inorganic P×10²	Specific activity relative to adrenal inorganic P×10²
Room temperature	15	26.6±1.4†	52.7±1.7	50.4±0.7
16 hours cold	7	46.8±3.1	89.0±4.0	68.9±2.8
P[1]		<0.001	<0.001	<0.001

* Injected dose, 4.3×10^7 cts./min. for a 200 gm. rat.
† Mean ± standard error.
[1] P value for animals in the cold compared with control rats at room temperature.

Adrenal Phospholipid

Table 2 shows that exposure of the animals to cold caused a significant increase in the specific activity of the lipid P. The specific activity of the lipid P relative to that of the inorganic P of either the plasma or the adrenal gland itself was also increased. The value obtained for the relative specific activity of the lipid P of the animals maintained at room temperature agrees with that previously reported from this laboratory (Riedel, *et al.*, 1954, 1954b).

In the interpretation of specific activity measurements it is important to know if the total amount of the constituent under study remained constant throughout the course of the experiment. Table 3 shows that for the lipid P this was not the case. During the 16 hours of cold exposure there was a significant ($P < 0.05$) increase in the concentration of lipid P in the adrenal. Although the cold exposure did not cause a significant change in

TABLE 3. EFFECT OF EXPOSURE TO THE COLD (3 ± 1° C) FOR 16 HOURS ON THE COMBINED ADRENAL WEIGHT AND THE CONCENTRATION OF LIPID PHOSPHORUS IN THE ADRENAL GLAND OF THE RAT

Group	No. determinations	Combined adrenal weight (mg.)	Lipid P (mg./100 gm.)	Total lipid P (μg./pr. adrenals)
Room temperature	15	27.3 ± 0.8*	149 ± 1.3	40.8 ± 1.2
16 hours cold	7	29.0 ± 0.8	157 ± 4.3	45.6 ± 1.3
Percentage increase		7.2%	5.5%	11.8%
P[1]		0.2–0.1	<0.05	<0.05

* Mean ± standard error.
[1] P value for animals in the cold compared with control rats at room temperature.

the combined weight of the adrenal glands, the mean figure was increased slightly, so that the percentage increase in total lipid P per pair of adrenal glands was greater than the percentage increase in the concentration of lipid P.

It might be considered surprising that exposure of an animal to cold for such a short period of time should cause a significant change in the concentration of lipid P. However, exogenous ACTH can cause equally rapid changes in the concentration of lipid P. Riedel *et al.* (1954, 1954b) found that the concentration of lipid P was decreased 48 hours after removal of the pituitary. In hypophysectomized rats an intraperitoneal injection of ACTH caused a significant increase within 20 hours.

The concentration of lipid P in the adrenals of the rats maintained at room temperature was higher than that reported previously (Riedel, *et al.*, 1954, 1954b), while the total amount of lipid P (per pair of adrenals) was lower. These differences may be explained by the fact that younger rats were used for the present investigation.

Adrenal RNA Nucleotides

Table 4 shows the effect of exposure to the cold for 16 hours on the incorporation of P^{32} into the four RNA nucleotides of the adrenal gland. In

TABLE 4. EFFECT OF EXPOSURE TO THE COLD (3 ± 1° C) FOR 16 HOURS ON THE RELATIVE SPECIFIC ACTIVITY OF THE RNA NUCLEOTIDES OF THE ADRENAL GLAND OF THE RAT

Group	No. determinations	Specific activity relative to adrenal inorganic P × 10^2			
		Adenylic acid	Guanylic acid	Cytidylic acid	Uridylic acid
Room temperature	13	35.7 ± 2.2*	32.8 ± 1.4	33.8 ± 1.4	33.3 ± 1.7
16 hours cold	6	57.3 ± 4.3	54.6 ± 4.0	55.6 ± 4.0	55.4 ± 7.5
P[1]		<0.001	<0.001	<0.001	<0.001

* Mean ± standard error.
[1] P value for animals in the cold compared with control rats at room temperature.

the cold-exposed animals the specific activity of each of the four nucleotides, relative to the specific activity of the adrenal inorganic P, was increased significantly. Since the specific activity of the inorganic P of the adrenal of the cold-exposed rats was shown to be greatly increased (Table 1), it follows that there was an even larger increase in the specific activity (in terms of counts/min./μg. P) of the RNA nucleotides. The percentage increases in the relative specific activities of the four nucleotides were similar, and nearly twice as great as that for the lipid P.

DISCUSSION

Phospholipid

The increase in the specific activity of the lipid P in the adrenal glands of rats exposed to cold may be the result of (a) an increase in the rate of renewal of lipid P, with no net synthesis of lipid P during the 16 hours of the experiment, (b) a net synthesis of lipid P during the experiment, with

TABLE 5. CALCULATION OF NET SYNTHESIS OF LIPID PHOSPHORUS NECESSARY TO ACCOUNT FOR OBSERVED INCREASE IN SPECIFIC ACTIVITY

	Room temperature	16 hours cold
Calculated mean specific activity of intracellular inorganic P (cts./min./μg. P)	130	189 (a)
Observed mean specific activity of lipid P from Table 2 (cts./min./μg. P)	26.6	46.8 (b)
Calculated specific activity of lipid P, if renewal rate was the same as for room temperature animals (cts./min./μg. P)		38.7 (c)
Calculated new lipid P that must be synthesized to account for increase in specific activity of lipid P, without any increase in renewal rate (%)		$5.7\% \left[\frac{(b-c)}{(a-b)} \times 100 \right]$

no change in the rate of renewal of lipid P already present, or (c) both an increase in the rate of renewal and a net synthesis of lipid P.

It is known from the data presented in Table 3 that exposing an animal to the cold for 16 hours causes an increase in the amount of lipid P in the adrenals expressed as μg. P per pair of adrenals. It remains to be shown whether sufficient new lipid is synthesized to account for the observed increase in the specific activity of the lipid P, or whether there is also an increase in rate of renewal of the lipid P already present.

Table 5 shows the mean specific activity of the adrenal intracellular inorganic P, evaluated graphically from Fig. 1, for both the room-temperature and the cold-exposed animals. Table 5 also shows that, if the renewal rate of the lipid P in the adrenals of the cold-exposed rats were the same as that of the control rats maintained at room temperature, the specific activity of the lipid P would be $(26.6 \times 189)/130$, or 38.7 counts per min.

per μg. P, instead of the observed 46.8. For the cold-exposed animals it may be calculated that the synthesis of an additional 5.7% new lipid P during the 16 hours of the experiment would account for the observed increase in specific activity of lipid P. In this calculation (details given in Table 5) the assumption is made that the lipid P synthesized during the experiment is formed in the adrenal gland from inorganic P, or from some compound in rapid equilibrium with inorganic P, with no rate-limiting reaction intervening, i.e. that the specific activity of the newly synthesized lipid P, as opposed to that of the lipid P already present and part of which is renewed, is the same as the mean specific activity of the intracellular inorganic P throughout the 16-hour period during which the animal was exposed to the cold. Although little is known of the biosynthesis of phospholipids in the adrenal gland, experiments in this laboratory[5] have shown that the phospholipids of the adrenal are labeled *in vitro* from inorganic P^{32}. For other tissues, some of the details of phospholipid formation have been elucidated (Kennedy, 1953; Kennedy and Weiss, 1955; Kornberg and Pricer, 1953).

The figure of 5.7% shown in Table 5 is considerably less than the 11.8% reported in Table 3 for the increase in the amount of lipid P in the adrenal during the 16-hour period, or the increase of 13.8% reported in Table 6. It may be concluded that the increased labeling of lipid P in the adrenal

TABLE 6. EFFECT OF EXPOSURE TO THE COLD (3 ± 1° C) FOR 16 HOURS ON THE CONCENTRATION OF LIPID PHOSPHORUS AND RIBONUCLEIC ACID PHOSPHORUS RELATIVE TO THAT OF DESOXYRIBONUCLEIC ACID PHOSPHORUS IN THE ADRENAL GLAND OF THE RAT

Group	No. determinations	Lipid P		RNAP		DNAP		Lipid P / DNAP	RNA / DNAP
		(mg./100 gm.)	(μg./pr.)	(mg./100 gm.)	(μg./pr.)	(mg./100 gm.)	(μg./pr.)		
Room temperature	12	151* ±2.8	42.1 ±3.1	45.4 ±2.4	12.6 ±0.7	49.4 ±2.1	13.7 ±0.7	3.11±0.14	0.95±0.08
16 hours cold	12	155 ±4.6	47.9 ±1.6	47.7 ±2.6	14.7 ±0.8	50.7 ±3.1	15.6 ±1.0	3.16±0.19	1.00±0.10
Percentage increase			13.8%		16.7%		13.9%		
P[1]		>0.4	0.2–0.1	>0.5	0.1–0.05	>0.7	0.2–0.1	>0.8	>0.7

* Mean ± standard error.
[1] P value for animals in the cold compared with control rats at room temperature.

glands of rats exposed to cold can be accounted for by the synthesis of new lipid P, and that there is no need to postulate an increase in the rate of renewal of the lipid P already present.

RNA Nucleotides

In experiments such as these, isolation of the RNA nucleotides is necessary, since the tissue RNA phosphorus, as separated by the usual procedures, contains a number of high-activity non-nucleotide phosphorus-

[5] Dennes, Elizabeth: unpublished observations.

containing compounds (Davidson, Frazer and Hutchinson, 1951; DeLuca, Rossiter and Strickland, 1953). Unfortunately, the method used for the isolation of the RNA nucleotides is not quantitative, although it yields a product suitable for specific activity measurements. In order to determine whether the exposure of the animals to cold for 16 hours caused the synthesis of new RNA, similar to the net synthesis of lipid P, further experiments were undertaken using the method of Schmidt and Thannhauser (1945) for the quantitative determination of RNA and DNA.

Table 6 shows that exposure of the rats to the cold for 16 hours caused no significant change in the concentration of lipid P, RNA or DNA. However, there was an increase in the mean amount of each of these substances when the results were expressed in terms of μg. P per pair of adrenal glands. The probability that these increases are the result of the cold exposure is relatively high ($P = 0.2$–0.05). In the previous experiments (Table 3), in which a larger sample of adrenal tissue was analyzed (9 pairs instead of 2 pairs), the increase in the amount of lipid P per pair of adrenals was significant at the 5% level. Also, in experiments still unpublished the increase in the amounts of lipid P and RNA per pair of adrenals in rats exposed to the cold for a longer period (8 days) was highly significant statistically ($P < 0.001$).

The possibility remained, therefore, that the increased specific activity of the ribonucleotides in the adrenals of rats exposed to cold, in a fashion similar to that already described for lipid P, might be attributed to net synthesis of RNA, rather than to an increase in the rate of renewal of ribonucleotide P already present. A calculation similar to that shown in Table 5 for lipid P, with similar assumptions, indicates that the synthesis of 7.9% new RNA would account for the increase observed in the mean specific activity of the RNA nucleotides in the adrenals of the animals exposed to the cold for 16 hours. This figure is considerably less than the mean increase of 16.7% reported in Table 6. Thus the increased labeling of the ribonucleotide P in the adrenals of rats exposed to cold, like the increase in the labeling of lipid P, can be accounted for by the synthesis of new material. There is no need to postulate an increase in the rate of renewal of ribonucleotide P already present.

Adrenal Phosphorus Metabolism in Cold-Exposed Animals

The results presented here confirm the finding of Nicholls and Rossiter (1955a) that there is an increase in the incorporation of P^{32} into the inorganic P of the adrenal glands of rats exposed to cold for short periods of time. It has been shown now that exposure of the rats to the cold for 16 hours causes an increase in the labeling of phospholipids and RNA nucleotides. In addition, there is an increase in the total amount of lipid P, RNA and DNA in the gland. The increase in the lipid P and the RNA is of the same order as the increase in the DNA. These increases are associated with

the adrenal hyperplasia known to occur in animals exposed to the cold.

The changes in the phosphorus metabolism of the adrenals of rats exposed to the cold for 16 hours are very similar to those that occur in the adrenals of hypophysectomized rats 20 hours after an intraperitoneal injection of ACTH (Riedel *et al.*, 1954a; Riedel *et al.*, 1954b; Logan *et al.*, 1955). This gives added strength to the suggestion of Nicholls and Rossiter (1955b) that the changes in phosphorus metabolism of the adrenal gland that follow short periods of cold exposure are due to the release of endogenous ACTH from the pituitary.

After reviewing the evidence, Logan *et al.* (1955) concluded that there are at least two changes in the phosphorus metabolism of the adrenal glands of hypophysectomized rats brought about by an injection of ACTH: (a) an increase in the rate at which inorganic P^{32} passes from the extracellular to the cellular portion of the gland and (b) an increase in the formation of organic compounds (e.g. lipid P and RNA nucleotides) from cellular inorganic P.

Nicholls and Rossiter (1955a) concluded that in animals exposed to cold for a short period of time there is an increase in the rate at which inorganic P^{32} passes from the extracellular to the cellular portion of the adrenal gland. In the present paper a net synthesis of P-containing compounds (lipid P and RNA nucleotides) has been demonstrated. These studies, taken in conjunction with others published from the laboratory, indicate that activation of the adrenal gland by either exogenous or endogenous ACTH results in a characteristic series of changes in the phosphorus metabolism. These probably indicate a change in the over-all metabolism of the gland. The primary site of the action of ACTH on adrenal metabolism is still unknown.

SUMMARY AND CONCLUSIONS

The specific activity of the inorganic phosphorus of the adrenal gland of rats maintained in a cold environment ($3 \pm 1°$ C) for the 16 hours immediately following the injection of inorganic P^{32} was greater than that of similar animals maintained at room temperature.

The specific activity of both the lipid phosphorus and the RNA nucleotide phosphorus was also increased, as was the specific activity of each of these fractions relative to that of the inorganic phosphate of either the plasma or the adrenal gland itself.

It is concluded that the changes in the phosphorus metabolism of the adrenal gland during a short exposure to a cold environment are brought about by the liberation of ACTH from the pituitary. These changes are: (a) an increase in the rate at which inorganic P^{32} passes from the extracellular to the cellular portion of the gland and (b) a net synthesis of phosphorus-containing organic compounds (e.g. lipid phosphorus, RNA nucleotides).

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CONCERNING THE FUNCTION OF THE FETAL ZONE OF THE HUMAN ADRENAL GLAND[1]

KURT BENIRSCHKE,[2] ERIC BLOCH[3] AND ARTHUR T. HERTIG[2]

Department of Pathology, Boston Lying-in Hospital and Harvard Medical School, Boston, Massachusetts and The Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts

THE unusually large size of the adrenal glands of the human fetus (Figs. 1, 2) and their rapid partial involution have been the subject of numerous reports and investigations. Lanman (1953) contributed an admirable summary of all known details concerning the development and probable function of this gland in the developing human fetus and added a valuable comparative anatomic study of the adrenals in other animals. While a wealth of contributions to the literature is cited by Lanman (1953), his conclusion is that little definite is known regarding the actual physiology of the adrenal in the unborn human fetus.

In this communication the authors have attempted to summarize the more important literature pertaining to the human fetal adrenal. In addition, a histologic correlation with the chemical data of the preceding paper is presented. On the basis of this material and evidence derived from the literature the endocrine relations are discussed which are of probable significance in the maintenance and function of the fetal zone of the human adrenal gland.

The large size of the fetal adrenal is principally due to the presence of a thick inner layer of cortical cells which is doomed to rapid involution upon birth. This inner layer of cortical cells has been named the "fetal cortex" by Elliott and Armour (1911). Gruenwald (1946) prefers the name "X-zone" and Grollman (1936), among others, has referred to this layer as the "androgenic zone." Parallels have been drawn between this tissue and the X-zone of mice and lower animals (Howard, 1927; see Lanman, 1953). From the review of Lanman (1953) and others (Benner, 1940; Blackman, 1946) it is obvious that this zone involutes with regularity upon birth more or less rapidly and this has been the experience of the present authors. In a morphologic study of the regressive changes of this zone, Blackman (1946) concludes that the involutionary process does not en-

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[2] Harvard Medical School, Boston, Mass.

[3] Worcester Foundation for Experimental Biology, Shrewsbury, Mass.

compass the entire mass of cells making up the fetal cortex but that some of its cells survive to become gradually transformed into the reticular zone of the adult cortex. Benner (1940), studying the same process, comes to the conclusion that the regressive changes begin, on occasion, before birth, a finding corresponding to our experience with some stillborn infants and infants dying in the immediate postnatal period. Involution proceeds

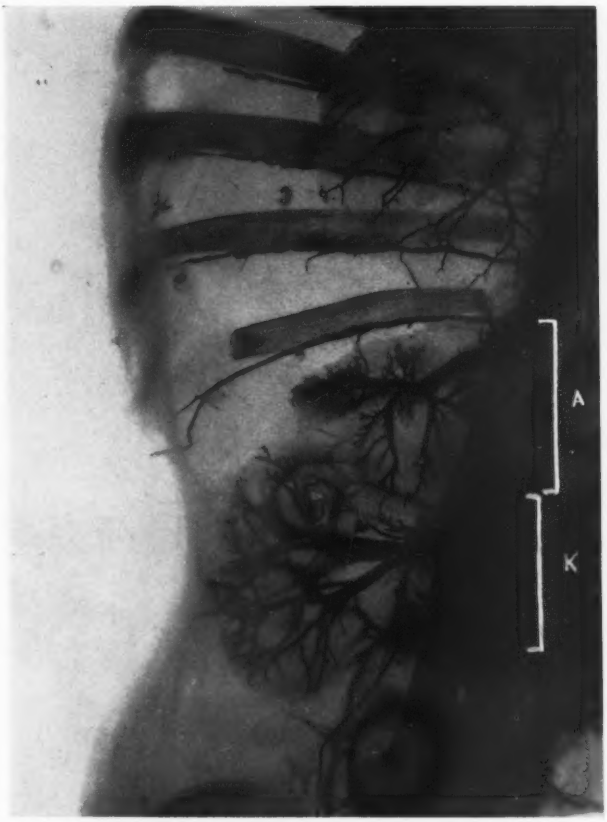

FIG. 1. Injection study of fetus measuring 10.5 cm. CR length (13 weeks gestational age). Note the large vascular tree above kidney (K) of right adrenal gland (A). (The liver has been partially dissected away.)

equally as rapidly in fullterm infants as in prematurely born babies. As Lanman (1953) indicated, there is considerable variation in the rapidity of the involutionary changes observed at postmortem examination and he cites instances of alleged delayed involution.

In an embryologic study, Uotila (1940) described the early formation of the glands. He observed a primary and secondary proliferative phase,

presumably giving rise to the fetal and definitive zones respectively. This is in accordance with the observation of Keene and Hewer (1927) on fewer specimens. Uotila's view, supported by other observers, of a coelomic mesothelial origin of the adrenal cells is at variance with Gruenwald's (1946) interpretation. The latter author holds that the cells forming the adrenal gland arise from the subcoelomic mesenchymal cells. Also, Gruen-

FIG. 2. Adrenal gland (A) and kidney of fetus measuring 10.5 cm. CR length. (BLiH S-55-1782, spontaneous abortion, 13 weeks gestational age.)

wald (1946) finds no distinct secondary cellular proliferative phase giving rise to the permanent (or true, adult) cortical elements. The distinctive histologic difference of the fetal and definitive layers, their different behavior in fat stains (Keene and Hewer, 1927) and studies by Greep and Deane (1949) and others lead us to conclude that these zones are of a different origin, nature and behavior. Throughout fetal existence the defini-

tive cortex remains small in amount compared to the fetal zone, only to proliferate after the latter has disappeared. During the last ten weeks of gestation there is a gradual increase in the thickness of the permanent cortex (Keene and Hewer, 1927), however, in the normal newborn infant the fetal zone still makes up approximately 80% of the thickness of the cortex (Bongiovanni, 1951; Lanman, 1953).

FIG. 3. Acardiac, amorphous twin at term. No adrenal tissue was found. (Courtesy: Dr. Philip LeCompte.)

In contrast to this normal behavior is the evolution of the adrenal gland in anencephalic conceptuses. The adrenal glands in such monsters are extremely small and, probably because of their small size, they have been reported as being absent on occasion by such competent observers as Gruber (1930) (1 absent in 26 cases), Meyer (1912) and others (Review by Bachmann, 1954). Angevine (1938) competently reviewed this subject and concludes that, while the adrenals are small, it is most likely that they are always present in anencephaly. In his 15 cases with "complete informa-

tion" a pituitary gland and the adrenals were found in every instance (in one case only 1 adrenal was found). Of interest is his finding of relatively larger glands in smaller anencephalic babies. This corroborates well with Meyer's (1912) observation of normal sized adrenals in anencephalic monsters of the 2nd and 5th month of gestation (1.4 and 13 cm. CR length). Meyer's (1912) histologic findings revealed that in such malformed fetuses

FIG. 4. Macerated acardiac, acephalic twin. (BLiH S-51-538, 3.8 cm. CR length, approximately 7 weeks gestational age.)

the adrenal cortex may be composed of its normal elements in the early stages of development but, for unknown reasons, it undergoes atrophy following the fifth month of gestation. Lipides were present chiefly in the "zona reticularis" (probably the fetal zone). The same was found true of a 25 mm. anencephalic described by Keene and Hewer (1927). This finding is at variance, however, with Pappenheimer's (1916) interpretation of a

single case in which he was unable to detect evidence of atrophy of a formerly existing fetal zone. In a subsequent communication (Benirschke, 1956A) the adrenal findings of our material of 39 anencephalics will be reported. At this point it may be remarked that our observations are in agreement with the descriptions of Meyer (1912), Keene and Hewer (1927) and Angevine (1938). A normal fetal zone may be found in the smaller anencephalic infant and this zone atrophies after approximately

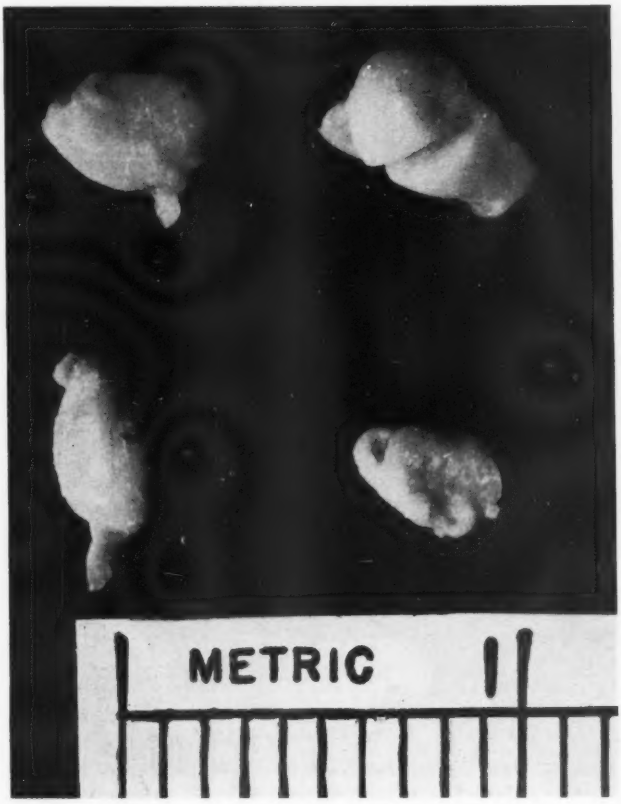

FIG. 5. Adrenals (top) and kidneys (bottom) of acardiac twin. (See Fig. 4, S-51-538.) Adrenals are of normal size and show histologically verified normal fetal zones.

20 weeks of gestation. In addition, it has been our experience that the premature involution of a fetal zone is not unique to the anencephalic at term but may also be observed in children with such anomalies as hydrocephaly, hemianencephaly and cephalomeningocele, provided there is destruction of or reduction in the size of the hypothalamus. In such cases, in contrast to the finding in the normal infant, chrome-alum hematoxylin staining

neurosecretory material was found absent from the posterior pituitary (Benirschke, 1956A; Benirschke and McKay, 1953).

To the best of our knowledge, well documented cases of complete congenital absence of the adrenal glands have only been recorded in instances of acardiac, acephalic and amorphous monsters (Fig. 3) (Review of litera-

FIG. 6. Adrenal gland of female pseudohermaphrodite with adrenogenital syndrome, age 51 days. Note hyperplastic reticular zone (fetal zone remnants, F) with involutionary changes. Definitive zone at top (D). (BLiH A-48-68, EMB, ×200.)

ture by Bachmann, 1954). This condition, by necessity, is only found in single ovum twins with vascular anastomoses between the normal and the abnormal partners. The circulation is maintained by the normal twin and it is reversed in the monstrous twin. Some of such malformed single-ovum twins may be found to contain adrenal glands when examined carefully. Gillim and Hendricks (1953) indentified "one rudimentary adrenal gland"

in their unusually large holoacardius (34 cm. CH length, 2440 gm.). Boulgakow (1926) found two suprarenals in his full-term acardiac monster. His brief microscopic description does not include the relative thickness of the various zones, however, his drawing indicates a very atrophied gland. We have found two normal sized adrenal glands and kidneys in our macerated acardiac, acephalic twin which measured 3.8 cm. in CR length

FIG. 7. Adrenal glands of newborn infants: *Top:* Hyperplasia of fetal zone in child with multiple congenital anomalies, including penile agenesis (BLiH A-52-78). *Middle:* Normal child, 32 weeks gestation. Death from cerebral hemorrhage (A-52-70 BLiH). *Bottom:* Anencephalic monster at term (A-52-74).

(Figs. 4, 5). Generally, these monstrosities are so markedly deformed (Fig. 3) or macerated that little knowledge exists of the microscopic appearance of their organs. Despite maceration, our 3.8 cm. CR specimen shows a recognizably normal structure of the fetal adrenal with a broad fetal zone and a thin layer of superficial definitive cortex (Benirschke, 1956A).

Hyperplasia of the adrenals in newborn infants has frequently been reported in female pseudohermaphrodites (Broster *et al.*, 1938; Wintersteiner 1941; McKenna *et al.*, 1942; Wilkins, 1949; Zuelzer and Blum, 1949; Wilkins, 1950; Harris and Scowen, 1951), macrogenitosomia precox (Wilkins, 1949; Wilkins, Fleischmann and Howard, 1940) and some other conditions (Francis, 1940; Bloch *et al.*, 1955; Benirschke, 1956B). The

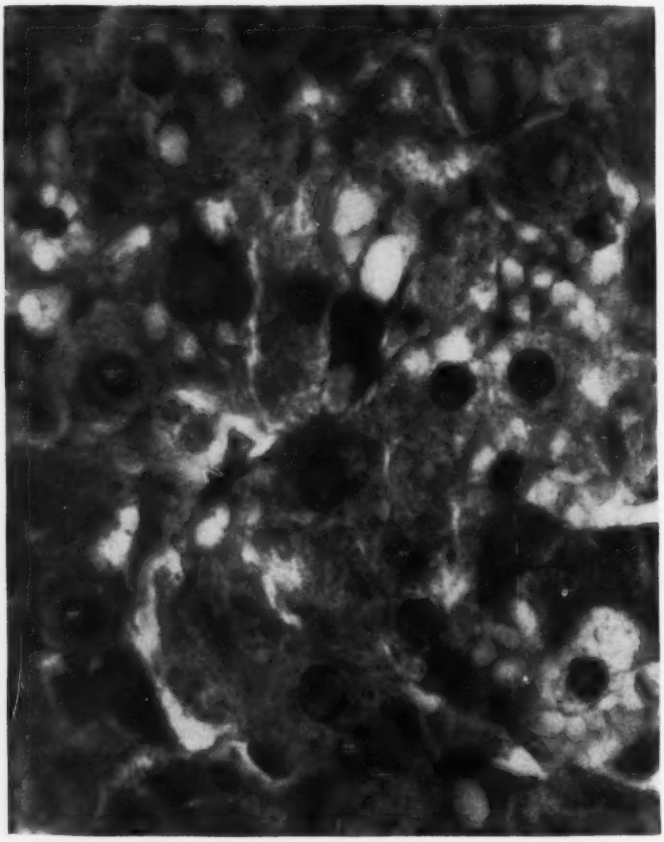

FIG. 8. Cells of fetal zone of A-52-78 BLiH. Hyperplasia of fetal cortex in case of multiple congenital anomalies, including penile agenesis. Compare with Fig. 17. (H&E, ×500.)

two former conditions are characterized by a hyperplasia of all elements of the adrenal cortex, with preponderance of one or the other zone. Involutionary changes may be observed in the fetal zone of such glands (Fig. 6). There is considerable variation from one case to another. In some, the definitive zone elements, more particularly the zonae glomeru-

losa and fasciculata, have been reported to be reduced in amount (Zuelzer and Blum, 1949; Wilkins *et al.*, 1940) and these frequently were found to be cases of more severe electrolyte imbalance of the Addisonian type (Blackman, 1946; Zuelzer and Blum, 1949). No histologic findings are available in Francis' case (1940) but an observation in this laboratory

FIG. 9. Hyperplastic adrenal cortex of A-52-78 BLiH. Child with multiple congenital anomalies including penile agenesis. Note hyperplastic fetal cortex has penetrated through definitive cortex (HF). The latter (D) is visible at right upper and lower corners, being markedly congested. (H&E, ×90.)

resembles his case very closely (Benirschke, 1956B). In our case the combined weight of the adrenal glands was 7.5 gm. (Fig. 7) and they had a remarkable appearance. The cells of the fetal zone were extremely large, granular and variable (Fig. 8). They made up the bulk of the gland and, similar to the case of Wilkins *et al.* (1940) (Case #5 in Blackman's (1946)

series), these fetal cortical cells had proliferated through the definitive cortex and extended, in areas, onto the capsule (Fig. 9). Unlike the case of Wilkins *et al.* (1940), there was an excessive amount of sudanophilic material (Fig. 10) in the fetal zone elements, which was accompanied by birefringence. Very little lipide was present in the cells of the small amount of definitive cortex, which showed remarkably congested sinusoids (Fig.

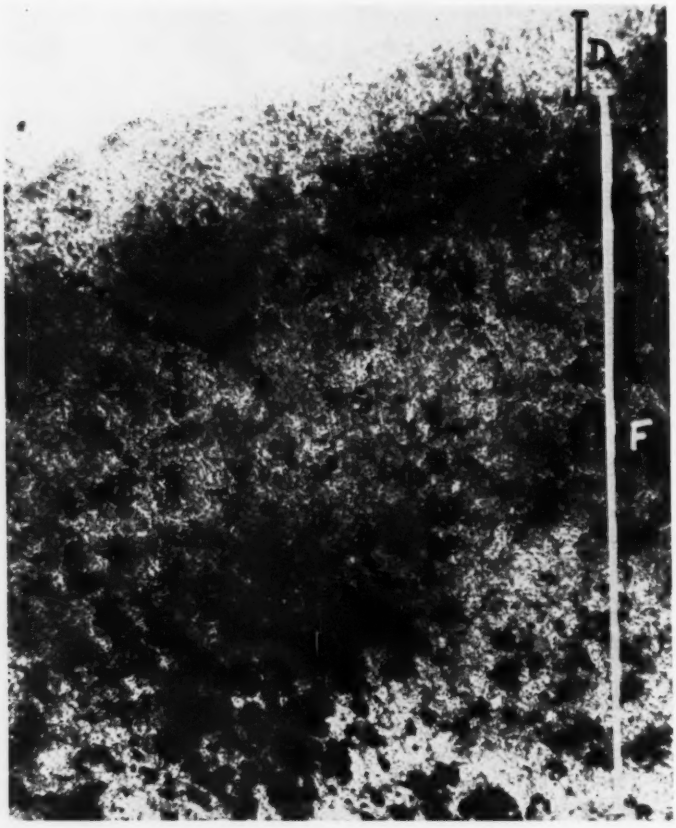

FIG. 10. Hyperplastic adrenal of A-52-78 BLiH. (See legend Fig. 9.) Lipides abundant in fetal zone (F), virtually absent in definitive layer (D). (Sudan-IV, green filter, ×90. Sudanophilic material appears black.)

9). This child died 5 minutes after delivery and autopsy was performed immediately. (Dx: Absence of penis. Hypoplasia of the kidneys,[4] hyperplasia of fetal zone of adrenals, myocardial infarct, adenomatous hyperplasia of lung, cerebral calcifications, excess of posterior pituitary neurosecretory

[4] In our previous communication (Bloch *et al.* 1955) this anomaly was printed as "hyperplasia of kidney," which is an error in printing.

material). Erroneously, the urogenital malformation was interpreted as probable pseudohermaphroditism (the child had testes). Since the original observation, the examination of a variety of sections of skin reveals the absence of "sex chromatin" (Moore *et al.*, 1953) and therefore, it is concluded that this child is genetically male. One of the adrenal glands was extracted for C_{19}-steroids and the results were presented as case #3 in a previous communication (Bloch *et al.*, 1955).

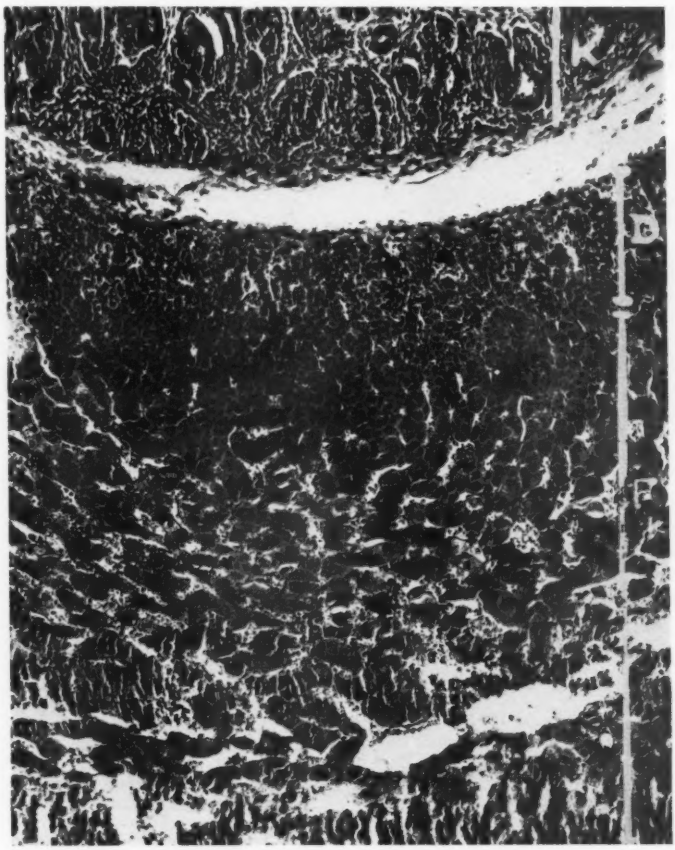

FIG. 11. Adrenal of normal fetus approximately 5.5 cm. CR length (10 weeks gestation). Therapeutic abortion for cardiac disease. (BLiH S-52-2039.) Kidney above (K), narrow definitive (D) and broad fetal (F) zones well distinguishable. (H&E, ×90.)

Extracts of adrenal glands of normal human newborns and fetuses had failed to yield steroid hormones with an androgenic property (Gersh and Grollman, 1939; Carnes, 1940). The following findings, however, presented arguments in favor of the occurrence of androgenic steroids in the fetal

zone of the human adrenal gland: The androgenic property of the extract obtained by Wilkins *et al.* (1940) in a single case of adrenal hyperplasia; the finding of a higher content of 17-ketosteroids in male and female human fetal bloods vs. maternal blood (Puck, Sievert and Meinecke, 1953; Gardner and Walton, 1954A); the greater content in 17-ketosteroids of newborn human urine, diminishing during the first few days of life

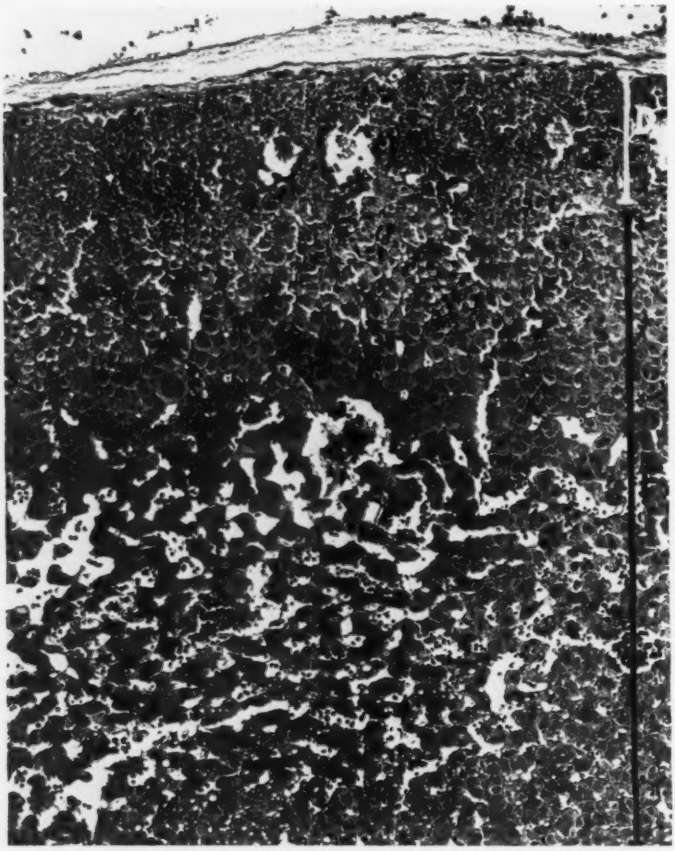

FIG. 12. Adrenal of normal male fetus 18 cm. CR length (19 weeks gestation age. S-52-1728 BLiH). Definitive (D) cortex above occupying approximately 1/5 of the width of the cortical tissue, 4/5 constitute fetal (F) zone. (H&E, ×90.)

(Read, Venning and Ripstein, 1950); the masculinization of female infants at birth in adrenal hyperplasia (Broster *et al.*, 1938; Wintersteiner, 1941; McKenna *et al.*, 1942; Wilkins, 1949; Zuelzer and Blum, 1949; Wilkins, 1950) as well as the abundant literature on the histochemical evidence suggestive of ketosteroid content of the adrenal glands (Recent review by

Deane and Seligman, 1953; Vines in Broster *et al.*, 1938; Freichels, 1952; Jones and Jones, 1954; Manly, 1955). The results of Puck *et al.* (1953) and Gardner and Walton (1954B) indicate a greater production of androgenic steroids (as measured by 17-ketosteroid content of umbilical cord blood) in younger fetuses vs. the older ones which would corroborate with

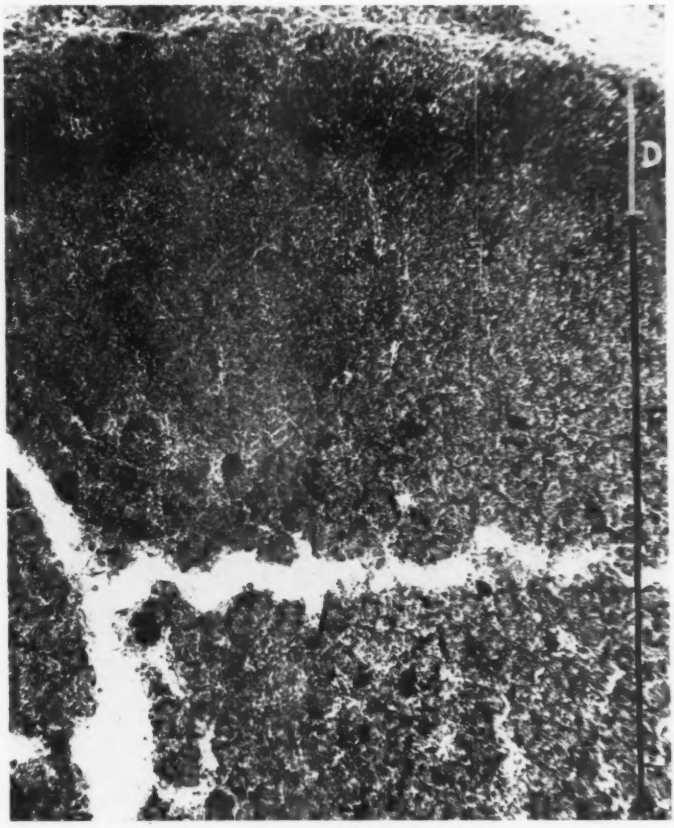

FIG. 13. Adrenal of normal fetus measuring 5.5 cm. CR length (10 weeks gestational age). Therapeutic abortion for uncontrollable diabetes mellitus. Lipides deposited in dense, finely granular form in the definitive cortex (above, D) and in large conglomerate droplets and fine granules through fetal zone (F), the latter constituting the majority of tissue and lipides. (BLiH S-52-2177. sudan-IV hematoxylin counterstain, green filter, ×90, sudanophilic lipides appear black.)

Vines' (Broster *et al.*, 1938) histologic interpretation of Ponceau-fuchsin stains of the gland.

The present authors thought it possible to clarify the situation by analyzing the extracts of a large number of immature adrenal glands obtained from fetuses at therapeutic abortions, realizing that androgen content not

necessarily indicates active secretion of such substances into the circulation (Severinghaus, 1942). The abortions were undertaken for the treatment of general diseases such as cardiac decompensation, tuberculosis, cancer, etc. and, to the best of our knowledge, there have been no patients in this group who were known to have previously delivered infants afflicted with the adrenogenital syndrome or other conditions affecting the adrenal

FIG. 14. Adrenal of normal premature infant measuring 22 cm. CR length, 557 gm. (22 weeks gestational age) survived 3 days. Abundant lipide deposits (black) in fetal zone (F), little present in definitive zone (D). (A-55-76, BLiH. Sudan-IV, hematoxylin, green filter, ×90.)

glands. In the stages examined (Figs. 11–14), the fetal adrenals are made up of predominantly fetal zone elements, and sudanophilic and birefringent material shows a preponderance in the juxtamedullary region (Figs. 13–16). No attempt was made to microdissect away the permanent cortex although this was considered and this may prove to become necessary in forthcoming incubation experiments. The identifiability of a C_{19}-keto-

steroid in 2 of 3 previously examined glands (Bloch *et al.*, 1955) showing abundant birefringent and sudanophilic material localized in the fetal zone of the cortex indicated a possible correlation between lipide, birefringence and the presence of steroids. It may be that these steroid hormones are in solution in the birefringent crystals (Greep and Deane, 1949) or the sudanophilic material may actually represent the steroid hormones

FIG. 15. Adrenal of fetus measuring 10.5 cm. CR (13 weeks gestational age, same as Fig. 2). (Sudan-IV, no counterstain, green filter, ×90, sudanophilic material appears black.)

themselves. Sayers (1950), reviewing the entire subject, finds a close parallelism between sudanophilia and cholesterol content and anterior pituitary regulation of these materials. However, he cites various authors differing with this observation and concludes that the matter deserves further study. Yoffey and Baxter (1950) made a careful analysis of the adrenal glands with respect to birefringence, sudanophilia and other histochemical

procedures. In the fresh-frozen gland there was no birefringence but it appeared in time with fixation and particularly after digitonin treatment. Sudanophilic material was closely associated with birefringent crystals but the latter did not take sudan dyes. Deane and Seligman (1953) have adduced the presently available evidence of correspondence between birefringent crystals, sudanophilia and steroid content of various organs.

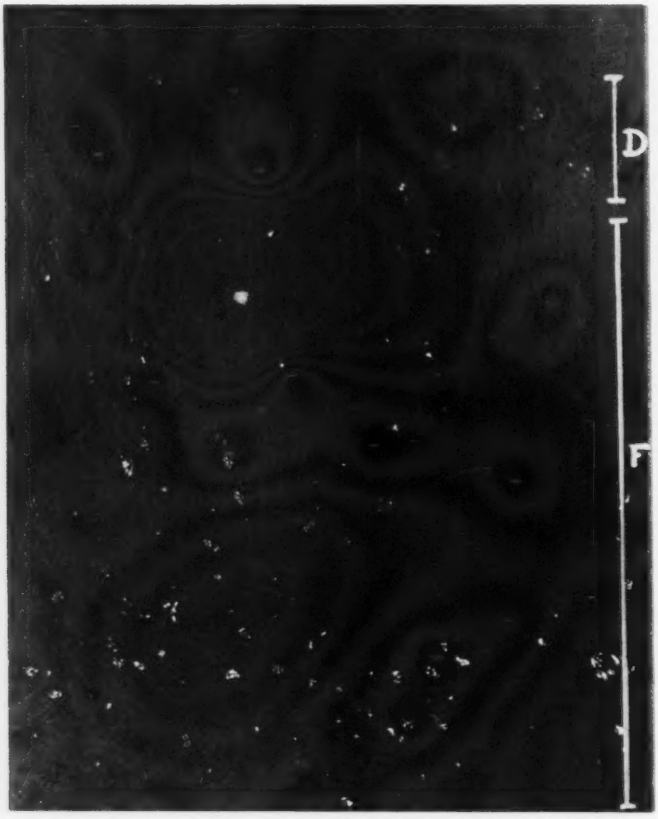

FIG. 16. As Fig. 15 but with partial polarization of light. Note correspondence of birefringent crystals with sudanophilic material in fetal zone (F) (×90).

This investigation was undertaken to show, if possible, the identity of androgenic steroids with the one found previously in one malformed and one normal infant (Bloch *et al.*, 1955) and to establish the nature of the incretory products of the normal fetal zone of the immature human adrenal gland.

Male and female human fetuses were separated, grouped according to ages and their adrenal glands were extracted and analyzed in a procedure

reported in detail in the preceding communication (Bloch *et al.*, 1956).

The isolation of C_{19}-steroid hormones, two of which having weakly androgenic properties, support the derivation of androgens from the fetal zone of the adrenal cortex as was suggested by the previosuly reported two cases (Bloch *et al.*, 1955). This finding agrees with most previous hy-

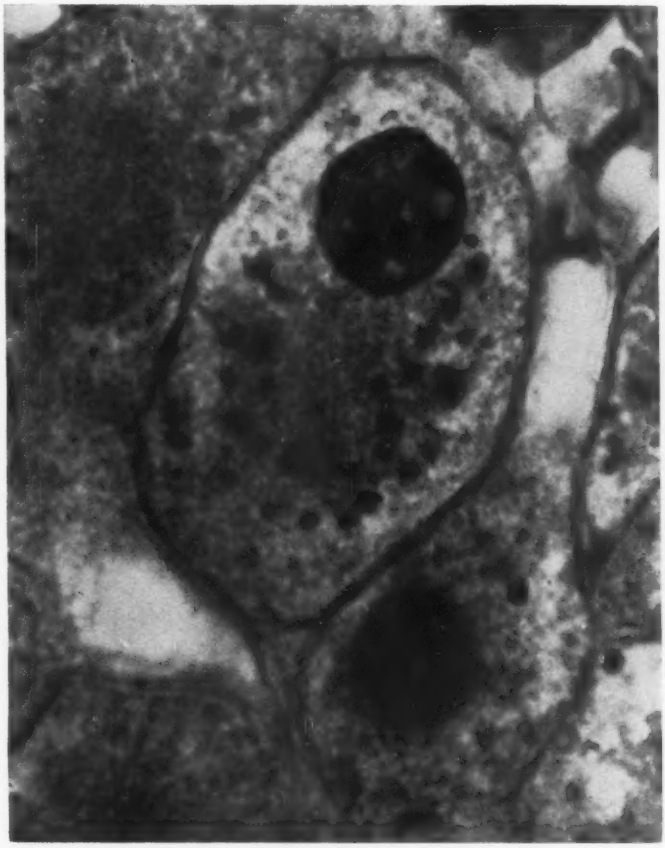

FIG. 17. Detail of cells of fetal zone in normal male fetus of spontaneous abortion measuring 10.5 cm. CR length (13 weeks gestation). Note similarity to cells of hyperplastic gland (Fig. 8). Prominent granules of purple color on light blue cytoplasmic background. (S-52-1904 BLiH. Gomori's chrome-alum-hematoxylin, ×1500.)

potheses and observations concerning the nature of this "androgenic zone" (Grollman, 1936). The approximately equal concentration of these androgens in the adrenals of female and male fetuses disagrees with the assumption of Vines (Broster *et al.*, 1938) of an "androgenic phase" in the development of the male from the 9th to 20th week and in the female from

the 11th to 15th week of gestation, based on Ponceau-fuchsin stains of the fetal adrenals.

The absence of 17-hydroxycorticosterone (Cortisol, Kendall's compound F) in the young fetus' adrenal and its later appearance supplement the recent findings of Staemmler (1953 A, B) of a gradual increase in the fetal adrenal content of C_{21}-steroids after the fifth month of gestation.

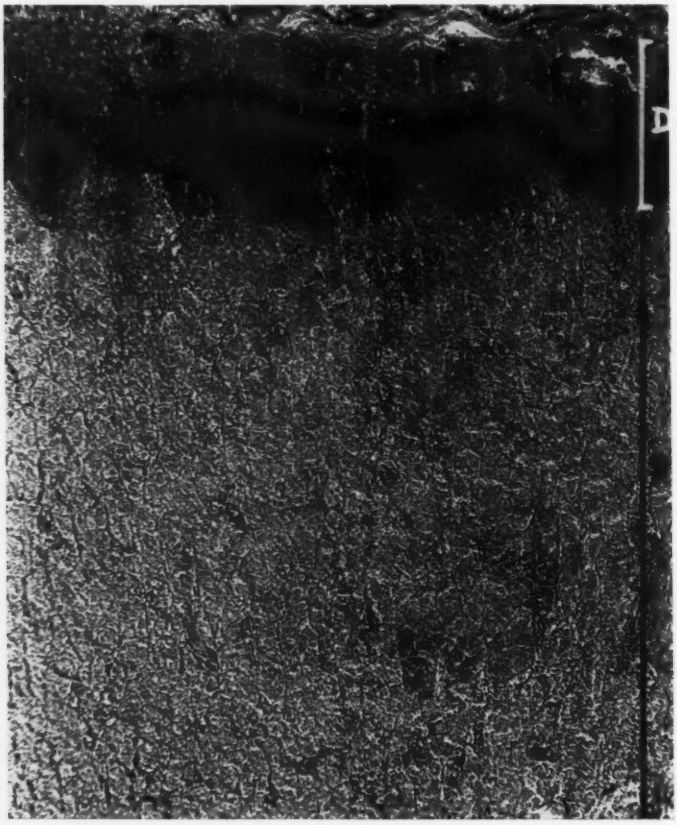

FIG. 18. Adrenal gland of stillborn infant at term with sacral meningocele. 40 weeks gestation. Large lipide deposits (black) in future zona fasciculata (D). (A-52-65 BLiH. sudan-IV, hematoxylin. ×90.)

These results suggest that the various layers of the fetal adrenal cortex are concerned with the production of individual steroid hormones and that these tissue layers respond to selective hormonal stimulation.

What is our evidence indicating the production of C_{19}-steroids by the fetal zone and C_{21}-steroids by the outer layers or the definitive cortex?

The androgen Δ^4-androstene-3,17-dione was the only steroid identified in a gland in which the fetal zone was hyperplastic (Bloch *et al.*, 1955) (Figs. 9, 10) and in an immature adrenal in which the majority of birefringent, sudanophilic crystals was localized in the fetal zone (Bloch *et al.*, 1955) (Case 1). Conversely, no steroids were obtained by the methods employed in a fullterm infant in which no birefringence was exhibited by the gland (Bloch *et al.*, 1955) (Case 2). There is some evidence favoring a correspondence between ketosteroids and birefringent lipides (Review by Deane and Seligman, 1953).

In the immature human fetus the vast majority of tissue forming the adrenal gland is fetal zone and the preponderance of birefringent lipide material is present in that region (Figs. 11–16).

In the young fetus only small amounts of granular, sudanophilic material are found in the definitive cortex (? containing sodium retaining steroids and small, immeasurable amounts of 17-hydroxycorticosterone). Toward maturity this zone widens (Keene and Hewer, 1927) and more lipide is accumulated in the outer portion of the gland (Fig. 18). Simultaneously, the extractable yield of C_{21}-steroids increases (Staemmler, 1953 A, B).

Additional evidence is the finding of C_{21}-steroids from the adrenal glands of four anencephalic monsters by Kloos and Staemmler (1953). In their cases, the amount of C_{21}-steroids extracted per unit of tissue was comparable to or higher than that of normal fetuses of the same gestational period. The weights given for these four cases indicate that the adrenals presented the usual atrophy of the fetal zone and was composed almost wholly of definitive cortex.

As pointed out above, there are diverse opinions concerning the correlation between lipides and steroid content. Lanman (1953) states: "The fetal zone is characteristically depleted of fat-staining material at the time of birth; while staining characteristics are not a reliable index of secretory activity, neither should the change in stored lipoid at birth be disregarded in evaluating the significance of postnatal steroid excretion." The fat content in adrenal glands, known to be hyperactive, has often been low (Wilkins *et al.*, 1940; Blackman, 1946). Deane *et al.* (1948) associate lipide depleted adrenal glands containing small cells with hypoactivity and lipide depletion with large cells with hyperactivity. Steege (quoted by Freichels, 1952) finds no lipides in the cells stained by the Ponceau-fuchsin method, in contrast to Zinsser and Zinsser (1951). The first occurrence of "lipines" in the fetal adrenal gland has been reviewed briefly by Uotila (1940). The earliest lipide to occur in human adrenals was demonstrated by Hett (1925) in a 23 mm. embryo.[5] It has been our experience

[5] Recently birefringent and sudanophilic material was seen in the adrenals of an 18 mm. embryo which had been fixed in formalin solution (McKay, D. G., personal communication).

that, in general, well preserved immature fetal adrenals have a lipide distribution as shown in Figs. 15 and 16. There is a fine granular sudan-IV staining deposit throughout the permanent cortex which gradually increases during gestation (Figs. 14, 18). The fetal zone contains irregularly scattered, large conglomerations of birefringent, sudan-IV staining material in addition to some scattered, more finely divided granular deposits (Figs. 15, 16). The amount of lipides in the latter zone varies considerably, generally it decreases toward term (Keene and Hewer, 1928) but it may be present at birth in large amounts as shown by Case #3 (Bloch *et al.* 1955) (Fig. 10). In younger fetuses (Fig. 14), the amount of sudan-IV staining lipides contained in the fetal zone is significantly greater than that of the permanent cortex. An additional amount can be stained by the sudan-black B method. Judging from these stains, the findings by extraction methods cited above and circumstantial evidence derived from the literature on the lipide depletion of other layers of the adrenal cortex in stress (Laqueur *et al.*, 1955; and others) and accumulation in prolonged cortisone therapy, there would appear to be some correlation between the lipide content and extractable steroids. Presupposing such correlation and corroborating our extraction results with lipide stains, it is apparent that the C_{19}-steroid production of the fetal adrenal gland is confined principally to the fetal zone vs. the C_{21}-steroid production being limited to the definitive cortex. This assumption, of course, is not new with us but may be found throughout clinical and pathological as well as chemical literature on steroid synthesis.

At this point it is speculative to state whether or not the finding of a higher concentration of androgens in the younger age group reflects an increased elaboration of such hormones. Evidence cited previously (Puck *et al.*, 1953; Gardner and Walton, 1954B) suggests that this may be the case. Incubation experiments now in progress will help to answer this question. Fig. 13 shows that in a fetus measuring 5.5 cm. CR a large amount of lipide, exhibiting birefringence is present in the fetal zone. If indeed younger fetuses were productive of larger amounts of androgenic steroids then a morphogenetic influence on the developing genital ducts, and possibly also the gonads, may be expected. The extremely rapid growth of adrenal elements to the very large size prior to the time of visual gonadal differentiation and prior to reduction and modulation of the sex ducts, in conjunction with their high phosphatase activity (Rossi *et al.*, 1951; McKay) favor such possibility. This is further supported by the often severe masculinization in female pseudohermaphroditism with adrenal hyperplasia. Wilkins (1949) has demonstrated clearly that, in some cases, such influence must occur at early stages of development (approximately the 11th week of gestation). From the voluminous literature on experiments concerning hormonal sexual differentiation it is evident that, most likely, such influence is exerted by permeation of steroids

rather than via the circulation (Willier, 1952; Grumbach *et al.*, 1955). The close proximity of the adrenal glands to the undeveloped genital ridge favors an interaction of the adrenal gland in the process of sexual differentiation if one is willing to accept the experimental data collected on the hormonal influence on this process. In the normal human development, however, it seems likely that differentiation of the sex ducts into the male direction is under the influence of testicular secretions (Philipp, 1951). The evidence for this may be found in the discovery of a genetically male nuclear pattern in the many cases of gonadal dysgenesis recently examined (Grumbach *et al.*, 1955). Frequently these patients were found to lack testes and present essentially normal external and internal female structures. In such cases, no evidence favoring congenital aplasia of the fetal adrenal cortex has been described as yet although urinary 17-ketosteroid excretion is said to be decreased (Mason and Engstrom, 1950). One such anomaly has been observed in this laboratory. The slightly prematurely born infant died at the age of 19 days. At autopsy the adrenal glands had a combined weight of 2.8 gm. and an approximately normal amount of fetal zone tissue was present. Moreover, our finding of equal concentrations of androgenic hormones in the adrenal glands of male and female fetuses negates a specific androgenic action of these hormones during the course of sexual differentiation.

It appears to the present authors that the chemical findings are best reconciled with the experimental evidence of a nonspecific "differentiator-action" (Willier, 1952) of androgens in the sexual differentiation of the pouch opossum (Burns, 1945). Beside this action, there may be other vital functions carried out by the fetal adrenal cortex during the development of the human embryo since complete lack of the adrenals apparently does not occur in human singletons.

The possibility of the elaboration of estrogenic hormones by the fetal cortex was considered in the past (Broster *et al.*, 1938), particularly in the case of the female infant. The possibility of such activity had been raised particularly because of the apparently shorter "androgenic phase" in the female adrenal cortex, as demonstrated by allegedly specific histochemical stains, and because of the sex and age relationships of the X-zone in the mouse. Carnes (1940) has determined the estrone content of the fetal adrenal glands (40 glands from the 18th–40th weeks of gestation) and found it to be of the same magnitude as that of other fetal tissues. In the analysis of our material no attention was paid to the possibility of the estrogen production of the fetal adrenal gland.

Factors probably concerned with the maintenance and activity of the fetal zone of the adrenal gland

In his final summary, Lanman (1953) has stated that: "First knowledge

of most endocrine organs has begun with recognition of a disorder associated with hyper- or hypo-function."

With our identification of androgenic steroid hormones in the human fetal adrenal gland, the demonstration of structural alterations during the development of anencephalic fetuses and since the recognition of endocrine factors of placenta and hypothalamus, a more plausible hypothesis for the endocrine relationship as it concerns the fetal adrenal gland may be advanced. It should be pointed out that some of the subsequent interrelations have been postulated by previous investigators on the speculation that the fetal zone of the adrenal is productive of progesterone or androgenic steroid hormones (Rotter, 1949; Moeri, 1951; Gardner and Walton, 1954A; Chang and Witschi, 1955).

The suspected cause of the uniform regression of the fetal zone at birth has been discussed in detail by Lanman (1953). His accumulated evidence strongly suggests that ACTH is not essential for the maintenance of the fetal zone in extrauterine life, indeed, administration of ACTH fails to prevent its involution. Moreover, the finding of an increased blood ACTH level in the adrenogenital syndrome (Sydnor *et al.*, 1953) with associated hyperplasia of all zonal elements in the presence of partial regression of the fetal cortex (Blackman, 1946; Zuelzer and Blum, 1949) (Fig. 6) further augment this conclusion. If a hormonal/trophic factor is active in stimulation and maintenance of this zone, and all available evidence points toward such a relation, then this factor must be disturbed in anencephaly (and the other pathologic conditions cited previously) and therefore it must reside in the pituitary or its hypothalamic connections. The occasional finding of an apparently normal (size and histology) anterior pituitary lobe in anencephaly (Angevine, 1938; Benirschke, 1956A) places the defect in the hypothalamus or the neurohypophysis. Our evidence (Benirschke, 1956A) of essentially normal development of the fetal zone in acephalic and anencephalic monsters (Figs. 4, 5) during their early stages of embryogenesis and the subsequent atrophy of this region clearly indicates that fetal pituitary-hypothalamus action on the maintenance of the fetal zone does not begin before the 20th week of gestation. This is in essential agreement with Meyer's (1912) observation and the postulations of other investigators. It is of interest to note that this critical time falls into the developmental period during which neurosecretory material is first being observed in the hypothalamus by Bargmann's (1949) method (Benirschke and McKay 1953). In the case of hydrocephaly, developing during the latter months of pregnancy, adrenal atrophy is due to hypothalamic destruction at this time (Benirschke 1956A).

No neurosecretory hypothalamic products have been described which exert a direct influence on the adrenal glands. Therefore, it would appear that such peptides (or nervous/vascular stimuli from the hypothalamus) react upon the anterior lobe of the pituitary gland to secrete a hormone

which then, in turn, influences adrenal function and regulates the size of the fetal zone. This anterior pituitary factor, operating during the second half of pregnancy, may either be ACTH or the gonadotrophins.

Evidence derived from Lanman's work (1953), the observation of high ACTH production in the adrenogenital syndrome and the 17-ketosteroid excretion pattern following the administration of ACTH (Mason and Engstrom, 1950) would indicate that there is some influence of ACTH on the fetal (and/or reticular) zone elements. For reasons given above, this cannot represent the only factor responsible for the maintenance of this zone, indeed, ACTH probably enhances physiologic involution in cooperation with the deprivation of the normal maintenance factor. The voluminous and frequently conflicting experimental data on adrenal gland maintenance by ACTH in hypophysectomized and decapitated fetuses of lower animals (Kitchell and Wells, 1952; Jost, 1949, 1950; and others) do not necessarily argue against this assumption since these species neither exhibit the exact same anatomic structure nor do they show regressive changes of the fetal adrenal gland at birth. Furthermore, the ACTH preparations used by these investigators may have been contaminated by small amounts of gonadotrophins although Kitchell and Wells (1952) present evidence of having worked with pure ACTH preparations.

Available data favor the interpretation that the luteinizing hormone (LH) represents the factor necessary for the maintenance and activity of the fetal zone of the human adrenal cortex.[6] In surgically castrated females there is a significant rise in the urinary 17-ketosteroid excretion following the administration of chorionic gonadotrophin (Botella-Llusiá and Nogales, 1953; Plate, 1953; Decio, 1953; Botella-Llusiá, 1953; Borell, 1954). Jones (1949) prevented X-zone degeneration in hypophysectomized mice by the administration of gonadotrophin, thought to contain mainly LH. ACTH and FSH failed to maintain this layer. Human chorionic gonadotrophin did not prevent degeneration. Botella-Llusiá and Nogales (1953) confirmed these results in similar experiments. (It must be borne in mind, however, that the X-zone of the mouse, despite histologic similarity, may not represent the precise homologue to the human fetal zone of the adrenal gland.) Gonadotrophins are produced in large amounts during the first months of pregnancy (Bruner, 1951) by the placental trophoblast (Waltz *et al.*, 1954) and the fetal tissues, as well as the amniotic fluid, contain amounts of gonadotrophins which are of probable biologic significance (Bruner, 1951). Chorionic gonadotrophin has a predominantly luteinizing property. It is during this stage of development that the fetal adrenal cortex of the anencephalic monsters is well developed. Subsequent to the significant decrease of chorionic gonadotrophins, the fetal cortex

[6] For the sake of simplicity the authors have abstained from discussing the evidence favoring the occurrence of only one pituitary gonadotrophin and participation of the lactogenic hormone.

involutes in the case of anencephaly while it is being maintained in the normal conceptus, presumably by the LH from the now active fetal pituitary gland. However, even in the normal conceptus there appears to be a quantitative reduction of circulating gonadotrophins with luteinizing property after approximately 4–5 months gestation, which presumably is responsible for a) the decreasing androgen production by the fetal adrenal; b) the minor atrophic changes observed in the fetal zone (Keene and Hewer, 1927) and c) the significant reduction of testicular interstitial cells (Gillman, 1948).

The stimulus for the pituitary secretion of LH has long been held to be the presence of large amounts of estrogens (Markee *et al.*, 1948; Bedoya and Jiménez, 1953). The latter steroids greatly increase during the second half of pregnancy (Breitner, 1954; Bradshaw and Jessop, 1953) and evidence of strong estrogenic stimulation of the genital tracts of both male and females may be found in many newborns as well as anencephalic monsters (Benirschke, 1956A). Markee *et al.* (1948) have proposed that the LH production of the anterior pituitary gland is dependent upon an intact hypothalamus and the work of Laqueur *et al.* (1955) strongly supports this view. This would explain the lack of LH production of the anencephalic, endowed with a normal anterior pituitary and showing apparent estrogen effect on the squamous epithelium of the genital system. The recent identification of an ACTH-releasing factor contained in the peptides extracted from hypothalami and neurohypophyses (Saffran, Schally and Benfey, 1955) leads one to expect a similar component which is responsible for the release of LH and which should be present in the human fetus. Further corroborating evidence comes from the demonstration of estrogen-induced adrenal hyperplasia in the larval frog (Chang and Witschi, 1955) which can only be obtained in nonhypophysectomized animals. These workers postulate an ACTH release, however. Finally, the paradoxical masculinization in experimental estrogen sex reversal studies (Burns, 1945) fits well into this scheme.

Thus it seems likely that the deprivation of estrogens at birth and shortly before delivery (Bradshaw and Jessop, 1953) resulting in reduced LH secretion causes the fetal zone to involute while the now initiated ACTH release causes the definitive zone to advance and secrete. This is augmented by the accumulation of C_{21}-steroids in the definitive zone of the fetal adrenal and their excretion within the first few days of life (Staemmler, 1953A; Klein *et al.*, 1954). Klein and Hanson (1950) and Gardner and Walton (1954B) suggested the inhibition of fetal pituitary ACTH production by placental corticosteroids. This is an attractive additional explanation of the various findings cited previously.

Finally, it is worth noting that the sodium-retaining activity found in the earlier fetus' adrenals may be of significant importance to the fetal homeostatic mechanisms. It may indicate a mechanism, additional to the

trophoblastic action by which the fetus is able to regulate its sodium and water retention during a developmental period when relatively low sodium and high potassium concentrations prevail (McKay *et al.*, 1955).

It is suggested that a very subtle endocrine balance exists between chorial and fetal hormones, necessary for the normal development of the human fetus. The theoretical probability exists that continued simultaneous estrogen and cortisone administration to the newborn fetus will maintain his fetal zone of the adrenal cortex.

SUMMARY

A brief review of the normal development of the fetal zone of the human adrenal gland is given and evidence presented indicating prenatal atrophy of this zone in the second half of gestation in anencephaly.

Chemical analysis of extracts of fetal adrenal glands show the occurrence of weak androgenic steroids and a sodium-retaining factor. The concentration of these steroids is higher in smaller fetuses and it is approximately equal in the male and female fetus. In older fetuses the glands contain small amounts of 17-hydroxycorticosterone.

The findings suggest that the C_{19}-steroidogenesis is limited to the fetal zone of the adrenal cortex vs. C_{21}-steroid production being performed by the definitive cortex.

The factors are discussed which are of probable importance for the maintenance of the fetal zone in intrauterine life. It is suggested that chorionic gonadotrophins are responsible for its maintenance during the first 20 weeks of gestation. Thereafter, luteinizing hormone from the fetal pituitary, stimulated by chorionic estrogens, is believed to replace the chorionic gonadotrophic stimulus.

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C_{19} STEROIDS, 17α-HYDROXYCORTICOSTERONE AND A SODIUM RETAINING FACTOR IN HUMAN FETAL ADRENAL GLANDS[1]

E. BLOCH, K. BENIRSCHKE AND E. ROSEMBERG

The Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts, the Department of Pathology, Boston Lying-in Hospital, Boston, Massachusetts, and the Worcester State Hospital "Dementia Praecox Research Project," Worcester, Massachusetts

THE embryological development of the human fetal adrenal cortex, and, perhaps, other primates, is unique. During the second month of fetal life, a distinct adrenal cortex is recognized. It reaches its largest relative size during the third and fourth month when it occupies about 0.5% of the total body volume as compared to 0.01% in the adult (Jackson, 1909). The histological picture of the fetal adrenal cortex differs from that of the adult by the presence of the fetal zone, which comprises about 80% of the whole cortex. The fetal zone degenerates rapidly during the first post-natal months. In addition, only the glomerular and fascicular zones of the cortex are fairly well developed in the fetus (Lanman, 1953).

In contrast to the available data on the morphology and anatomy of the human fetal adrenal cortex, its physiology remains largely unknown. An excellent review of the subject has been published by Lanman (1953). Large amounts of ascorbic acid (Bourne, 1933) and lipid material (Lanman, 1953) have been demonstrated by histochemical means to be present in the fetal zone. Staemmler (1953) observed increasing amounts of adrenal corticoids as fetal age progressed from the fifth lunar month to birth. Bloch *et al.* (1955) found the androgen Δ^4-androstene-3,17-dione in two human fetal adrenals, one being hyperplastic. The authors are not aware of any other chemical evidence for steroid production.

This study was undertaken to investigate the C_{19} and C_{21} steroid content in adrenal cortical tissue of human fetuses.

EXPERIMENTAL

Fetal Adrenal Glands: Adrenal glands were obtained from normal male and female fetuses, and kept stored individually in the frozen state. A detailed description of the physiological state and the source of these adrenals is given elsewhere (Benirschke *et al.*,

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1956). Prior to steroid analysis, the individual adrenals were pooled in groups according to sex and age (See Table 1).

Extraction and Purification: Each group of adrenals was homogenized in a Potter-Elvehjem homogenizer at 4° C with 3 volumes of cold, physiological saline solution. The homogenates were extracted repeatedly with redistilled ethyl acetate (2 v/v) and with diethyl ether-chloroform (4:1) (2 v/v), followed by the addition of 10 volumes of hot acetone to the aqueous residue. After standing for two hours, the supernatent was decanted, and the protein residue washed twice with 2 volumes cold acetone. The extract, after acetone removal, was extracted with ethyl acetate and ether-chloroform (4:1). The organic solvent extracts from the original homogenate and from the acetone extract were combined according to group of adrenals, and the solvents removed by distillation in vacuo at 50–56° C.

The dried extracts were partitioned between 90% methanol and heptane to remove fatty material. The methanol fraction was taken to dryness; the residue taken up in chloroform-ether (1:1); washed once with 0.1 N NaOH (0.2 v/v), and washed with water until neutral. After removal of water with anhydrous sodium sulfate, the solvents were removed by distillation and vacuum desiccation.

Chromatography: Silica-gel column chromatography of the extracts resulted in fractions 1 (100 ml. benzene), 2 (2×100 ml. benzene:ethyl acetate (2:1)), and 3 (2×100 ml. ethyl acetate:methanol (4:1)).

Fraction 2 was chromatographed twice on Whatman filter paper No. 1 in the solvent system heptane-propylene glycol (Savard, 1953), and in toluene-propylene glycol (Burton *et al.*, 1951). *Fraction 3* was chromatographed twice in toluene-propylene glycol and once (overflow, only) in heptane-propylene glycol. The area on the chromatograms of fraction 3 corresponding to the approximate mobility of aldosterone was eluted and examined for sodium retaining activity by bioassay.

All chromatograms were examined for the presence of Δ^4-3-ketosteroids (ultraviolet light absorbancy), 17-ketosteroids (Zimmermann reagent, Savard, 1953) and 21-ketolic steroids (blue triphenyl tetrazolium reagent (Burton *et al.*, 1951)). The reactive zones were eluted with methanol-chloroform, and aliquots taken for quantitative determinations. The appropriate eluates were pooled, and admixture paper chromatography (chromatography of eluate mixed with an authentic sample of the suspected steroid) carried out.

The bioassay method of sodium-retaining activity, a modification of the one developed by Marcus *et al.* (1952) employs the bilaterally-adrenalectomized male rat weighing between 135–150 g. Each animal was injected intraperitoneally with 45 mg. of sodium chloride and the urine was collected for 6 hours. The urinary sodium was determined by flame-photometry using a Perkin-Elmer Model 52C instrument and an internal standard of lithium. The eluted zones were assayed at a single concentration (20% ethanol), and the standard desoxycorticosterone at 6 and 25 μg. dosage levels were assayed simultaneously with the unknowns. 4 animals, grouped in pairs, were employed for the unknowns, and 8 animals, grouped in pairs, were employed for the solvent control and desoxycorticosterone standard.

RESULTS AND DISCUSSION

Three Zimmermann reactive 17-ketosteroid zones (purple color) were observed on the paper chromatograms of the extract from each group of fetal adrenals. Zones 1 and 3 absorbed ultraviolet light, indicating the presence of Δ^4-3-ketosteroids. The chromatographic mobility of zone 1 was identical with that of a standard reference sample of Δ^4-androstene-3,17-

dione (1.82–2.16 cm./hr. in heptane-propylene glycol), of zone 2 with dehydroepiandrosterone (0.88–1.14 cm./hr.), and of zone 3 with Δ^4-androstene-11β-ol-3,17-dione (2.5–3.1 cm./hr. in toluene-propylene glycol). Admixture chromatography resulted in a single zone in every instance. Group VI contained traces of 2 additional steroids, corresponding in their chromatographic behavior to Δ^4-androstene-3,11,17-trione and androstane-3,17-dione. Groups III and VI contained, in addition, 17α-hydroxycorticosterone.

The identification of these C_{19} steroids and hydroxycorticosterone is tentative. The criteria employed do not permit differentiation between dehydro*epi*androsterone, *epi*androsterone, and etiocholanolone, however, the following considerations offer strong confirmatory evidence for the correctness of identification. (a) The majority of steroids isolated from adrenal tissue have been 3-ketosteroids (Dorfman and Ungar (1953)). (b) With the single exception of androstane-3β,11β-diol-17-one (Reichstein and v.Euwe, 1938), all C_{19} steroids formed by adrenal tissue including fetal adrenals, have been 3,17-diketosteroids (Bloch *et al.*, 1955a; reviews by Roberts and Szego, 1955, and Dorfman and Ungar, 1953). (c) The production of dehydro*epi*androsterone by human adrenal tissue has been established (Bloch *et al.*, 1956).

The quantities of each C_{19} steroid found are presented in Table 1. A marked decrease occurs in the concentration of all three compounds after the 13th week of gestation. Increased secretory activity (commencement-?), reduction in synthesis, and/or a more rapid weight gain as compared to increase in steroid synthesis may explain the observed reduction. Sex differences and differences in both the absolute and relative amounts between the three 17-ketosteroids indicated by the data of Table 1, with the exception of the above noted decrease, are not considered significant.

The extracts from all groups of fetal adrenal glands exhibited sodium retaining activity (Table 2). The data represents results from male and female extracts pooled according to gestational age. In view of the small number of rats used per sample in the bioassays, it is not deemed advisable to express the results in terms of μg. equivalents of desoxycorticosterone. The suggestion is made on the basis of approximate chromatographic mobility that the observed sodium retaining activity may be due to a substance similar to or identical with aldosterone.

The trace amounts of 17α-hydroxycorticosterone detected in the adrenal extracts of Groups III and VI, and the absence of glucocorticoids otherwise stands in marked contrast to findings in adult human adrenal glands where 17α-hydroxycorticosterone and corticosterone are the major adrenocortical secretory products (Roberts and Szego, 1955). On the other hand, our results extend Staemmler's study (1953) of the presence of corticoid in older fetuses (20 weeks to birth) to fetuses less than 22 weeks old. As determined by the blue-tetrazolium color reaction on paper, the adrenal glands from

both male and female fetuses, 16.5–21.0 weeks old, contained about 0.8 μg. 17α-hydroxycorticosterone and corticosterone per gram tissue. Adrenals from younger fetuses contained less than 0.2 μg. hydroxycorticosterone or corticosterone. Hence, the ratio of the sum of the C_{19} steroids (as computed from Table I), to 17α-hydroxycorticosterone+corticosterone decreases with advancing age (9.5th to 21st week) from the order of 100:1 to 5–10:1.

The findings reported here would suggest that the early human fetal adrenal cortex (≦9th to 20th week of gestation) synthesizes primarily weak androgens. The radical difference in the ratios of androgen-glucocorticoid production between the early fetal and adult adrenal cortex, and the presence of detectable amounts of glucocorticoids after midgestation, would point to the fetal zone as the site of androgen production, and the fascicular zone as the locale of glucocorticoid synthesis. Alternately, the observa-

TABLE 1. 17-KETOSTEROID CONTENT OF NORMAL HUMAN MALE AND FEMALE FETAL ADRENAL GLANDS

Group	Length in cm. CR*	Gestational age (weeks)	No. of adrenals per group	Av. wt.† per adrenal gland (gm.)	17-Ketosteroid concentration (μg./gm. tissue)		
					Δ⁴-androstene-3,17-dione	Dehydroepiandrosterone	Δ⁴-androstene-11β-ol-3,17-dione
Males							
I	7.5– 9.5 cm.	11.0–12.5	17	0.1	6.0	6.4	4.7
II	10.0–12.0 cm.	13.0–14.5	16	0.2	2.6	3.7	4.8
III	15.0–19.5 cm.	16.5–21.0	24	0.6	1.5	1.7	0.4
Females							
IV	5.5– 8.2 cm.	9.5–11.5	10	0.1	14.1	8.5	7.3
V	10.5–14.5 cm.	13.5–16.5	34	0.2	1.9	4.0	2.1
VI	15.0–18.0 cm.	16.5–19.5	18	0.4	2.3	2.9	1.6

* CR (or Crown-Rump) length is the sitting height of the fetus and from it the gestational age was estimated by subtracting 2 weeks from the menstrual ages, calculated by Streeter (1920).

† These glands were dissected immediately following therapeutic interruption of pregnancy and frozen, stored at −30° C and collected over a 2 year period. During the process of dissection and weighing and while being stored, there has undoubtedly occurred some change in the weight due to dehydration. Hence, the values given here are to be interpreted as approximate ones.

tions of this investigation could be interpreted on the basis of selective storage of C_{19} steroids and sodium-retaining factors by the adrenal with a rapid secretory rate of glucocorticoids. Such a phenomenon has not been observed in any other instance of adrenal activity, and, hence, seems improbable. Finally, it is noteworthy that the adrenal cortex contains a sodium-retaining factor (aldosterone-like) as early as the 9th–11th week of gestation.

The preceding discussion, the results reported in the literature by other investigators (Benirschke *et al.*, 1956; Blackman, 1946; Lanman, 1953; Roberts and Szego, 1955; and others), and the contributions from studies of the adrenogenital syndrome (Wilkins, 1955), substantiate the hypothesis that adrenal androgens are synthesized in the fetal and reticular zones, glucocorticoids in the fascicular zone, and mineralocorticoids in the glomerular zone. For reasons described elsewhere (Benirschke *et al.*, 1956), it is considered that the production of C_{19} steroids by the fetal zone is controlled by lutenizing hormone (LH). The source of LH during early gestation is

TABLE 2. SODIUM RETAINING ACTIVITY IN FRACTION 3 FROM SILICA-GEL COLUMN CHROMATOGRAMS OF NORMAL MALE AND FEMALE FETAL ADRENAL GLAND EXTRACTS POOLED ACCORDING TO GESTATIONAL AGE

	Control	Desoxycorticosterone standard (μg.)		Fraction 3					
				Chromat. 1*			Chromat. 2*		
				Gestational age (weeks)					
		6	25	9.5 –12.5	13.0–16.5	16.5–21.0	9.5 –12.5	13.0–16.5	16.5–21.0
Urine volume (ml.)									
Mean	5.1	2.1	1.5	2.6	6.7	2.7	3.3	2.0	1.5
Range	4.0 –5.5	0.8 –3.2	1.1 –1.8	1.0 –4.1	6.0 –7.5	1.0 –4.5	2.0 –4.5	1.0 –3.0	1.0 –1.5
Sodium Excretion (mg./100 gm./hr.)									
Mean	1.68	0.87	0.49	0.95	1.77	1.20	1.23	1.13	0.82
Range	1.34–1.88	0.55–1.35	0.42–0.58	0.65–1.15	1.69–1.86	0.86–1.55	1.06–1.41	1.05–1.40	0.82
N	4	4	4	2	2	2	2	2	2

* = fraction 3 was chromatographed in toluene-propylene glycol for 25 hrs. (Chromatogram 1). A section of chromatogram 1 was eluted and rechromatographed in the same solvent system (chromatogram 2). The areas of both chromatograms corresponding to the mobility of aldosterone were assayed.

maternal chorionic gonadotrophin. After midgestation, the fetal pituitary, under the stimulation of increasing maternal estrogen levels, produces its own LH. Cessation of maternal estrogen action on the fetal pituitary, concomitant with birth, may be the cause of the marked decline in C_{19} steroid synthesis as judged by plasma and urinary 17-ketosteroid levels (Gardner and Walton, 1954a, 1954b; Read *et al.*, 1950). Insofar as the fetal zone is the site of androgen synthesis, its involution (caused by lack of maternal estrogens-?) would automatically be accompanied by cessation of androgen synthesis. The virtual absence of both the fetal and reticular zones, except for a narrow band of cells located close to the fascicular zone, until the third year (Blackman, 1946), explains the very low levels of urinary $C_{19}O_2$ steroids observed in infants and young children. From these findings, it is postulated that adrenal androgen synthesis is minimal during this period. With development and growth of the reticular zone through about the 18th year, C_{19} synthesis is again elevated to finally reach maximum levels during the second and third decades of life (Dorfman, 1948; Pincus *et al.*, 1955). In later life, androgen production declines (Pincus *et al.*, 1955).

The lack of demonstratable glucocorticoids in early fetal adrenal tissue may be due to (a) a sub-minimal ACTH production in and/or release by the pituitary per se; (b) inhibition of ACTH synthesis or release by circulating maternal corticoids, or (c) a lack of physiological conditions or enzyme systems essential for glucocorticoid synthesis within the fascicular cells. The observation of a slow and steady rise in glucocorticoid elaboration from midgestation to adult levels at the age of 3–7 years, without a marked change at birth (Heard, 1948; Read *et al.*, 1950; Staemmler, 1953; Zeisel, 1953) correlated with the development of the fascicular and glomerular zones—presence of both zones from early fetal life onwards, lack of regression at birth, and moderate enlargement during the first several years of postnatal life (Benirschke *et al.*, 1956; Blackman, 1946; Lanman, 1953) —strengthens the view that these zones are the sites of glucocorticoid synthesis. Responsiveness to ACTH stimulation and some histochemical evidence (Josimovich *et al.*, 1955; Jones, 1949; Yoffey, 1953) favor strongly the fascicular zone as the probable site of synthesis. Urinary excretion studies indicate that glucocorticoid synthesis remains fairly constant through the 7th decade of life (Pincus *et al.*, 1955).

Mineralocorticoid synthetic activity commences very early in the fetus and persists throughout life. The glomerular zone has been implicated as the site of elaboration (Dean and Seligman, 1953; Jones, 1949). Very little is known about the site of production of estrogens by the adrenal cortex. The ability of the adrenal gland to synthesize estrogens has been demonstrated by the isolation of estrone from bovine adrenal tissue (Beall, 1940), and the conversion of Δ^4-androstene-19-ol-3,17-dione to estrone by adrenal glands (Meyer, 1955).

It must be emphasized that the concepts developed above are largely

theoretical and depend to a considerable extent on evidence obtained from indirect studies of adrenocortical function (e.g., urinary steroid excretion). The authors believe that species differences do not admit of an interpretation of human adrenal function and development, particularly in the case of the fetal adrenal gland, on the basis of results obtained from studies with non-human species. Further, to date, histochemical studies have resulted in essentially opposing theories on the function of the reticular and fascicular zones (Yoffey, 1953; Yoffey, 1955; Symington *et al.*, 1955) which have provided much valuable data but have not elucidated the relationship between morphology and function. Extensive chemical studies on human adrenal cortical function, especially during the fetal and infancy-childhood age span, remain to be carried out to provide much needed experimental clarification.

SUMMARY

Adrenal tissue from normal, male and female human fetuses, from the 9th–21st week of gestation, were analyzed for their C_{19} and C_{21} contents. Three C_{19} steroids were tentatively identified by paper chromatographic methods as being present in all sex and age groups: Δ^4-androstene-3,17-dione, dehydro*epi*androsterone, and Δ^4-androstene-11β-ol-3,17-dione. Hydroxycorticosterone was detected in the tissue extracts from the 16.5–21.0 week of gestation. Extracts from all groups, when analyzed for sodium retaining activity by bioassay, were found to be active.

The concentration of C_{19} steroids per gram of adrenal tissue decreased with advancing fetal age (from about 30 to 8 μg./gm. tissue) without significant differences between the sexes. This decrease is ascribed to an (a) increased release by the adrenal gland, (b) reduced C_{19} steroid synthesis, or/and (c) larger adrenal weight increase than steroid synthesis.

The ratio of androgenic to glucocorticoid steroid content varied from a magnitude of 100:1 for the 9.5–12.5 week old fetal adrenals to 5–10:1 for the 16.5–21.0 week old fetal adrenals.

It is suggested that the early fetal adrenal cortex synthesizes primarily weak androgenic steroids and a sodium retaining factor similar to aldosterone. C_{19} steroid synthesis is considered to occur in the fetal zone with decreasing activity as fetal age advances. The hypothesis is advanced that the fetal and reticular zones are the sites of adrenal androgen production, and the fascicular and glomerular zones, respectively, the sites of glucocorticoid and mineralocorticoid synthesis, respectively.

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RADIOIODIDE IN THE THYROID AND IN OTHER ORGANS OF RATS TREATED WITH LARGE DOSES OF PERCHLORATE[1]

N. S. HALMI, R. G. STUELKE[2] AND M. D. SCHNELL[3]
with the assistance of D. A. WOODCOCK[4] and R. J. SWAN
Department of Anatomy, State University of Iowa, Iowa City, Iowa

WE INVESTIGATED the hitherto largely unexplored first step of thyroid hormone biogenesis, the diffusion of iodide into the thyroid, by administering radioiodide after complete blocking of the thyroidal iodide pump ("trap") with its most potent inhibitor, sodium perchlorate (Wyngaarden *et al.*, 1952). We correlated the amount of radioiodide that was found in the thyroid under such conditions with the structure of the gland, which was experimentally altered by varying the intensity of thyrotrophic stimulation. It was our hope that such a study may yield information concerning a) the site of the iodide "trap" within the thyroid parenchyma and b) the possible influence of thyrotrophin on the permeability of the thyroid cell to iodide. Levine and Goldstein (1955) have recently emphasized, in discussing the mode of action of insulin, that hormones may regulate metabolic processes by altering the permeability of cell membranes to specific substances.

Our investigations were then extended to the effect of perchlorate on a) the distribution of radioiodide in organs other than the thyroid, b) the radioiodide space of the whole body, c) the gastric iodide pump, d) intestinal absorption of radioiodide and e) excretion of radioiodide.

MATERIALS AND METHODS

1. *Experimental animals*

This study was performed on young adult male rats of the Sprague-Dawley strain which were maintained on Rockland pellets and tap water.

2. *Injections*

Injections were given subcutaneously.

a) *Propylthiouracil (PTU).* All animals received PTU to prevent organic binding of radioiodine. Unless otherwise specified, a single injection of 6 mg. of PTU in solution form was given.

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b) *Perchlorate.* Experiments on rats with highly active thyroidal iodide "traps," the results of which are included in Table 1, showed that 100 mg. of $NaClO_4$ was as effective as 200 or 400 mg. in abolishing the function of the iodide concentrating mechanism. Results obtained with these three dosages were therefore pooled in preparing Table 1, and in subsequent experiments 100 mg. of $NaClO_4$ was used.

c) *Chloride.* NaCl was given in a dose (47.5 mg.) equal to 100 mg. of $NaClO_4$ on a molar basis to rats which served as controls to the perchlorate-treated animals.

d) *Stable iodide.* When NaI was administered, the dosage (122 mg.) was also the molar equivalent of 100 mg. of $NaClO_4$. In one experiment (Table 1) 100 mg. of NaI was administered.

e) *Treatments designed to alter thyroid structure* are described in detail in the footnotes to Table 1. PTU was given together with TSH since it is known to enhance the effectiveness of this hormone (Halmi and Spirtos, 1954). Prolonged treatment with PTU was instituted to produce goiter. It was followed by three triiodothyronine injections in order to depress the activity of the stimulated iodide "trap" without abolishing the goiter. This was considered necessary because we assumed that only toxic or lethal doses of perchlorate may block the thyroidal iodide "trap" completely if the thyroid-serum radioiodide gradient is very high, as it is likely to be after chronic PTU administration. Four-hundred mg. of $NaClO_4$ was found to be close to the LD_{50}.

f) *Radioiodide.* Carrier-free I^{131} was given in doses ranging from 5 to 50 μc.

g) *Schedules.* When the rats were killed 1–1½ hours after the administration of radioiodide, the schedule of injections was as follows:

0 minute : 6 mg. PTU + 50 mg. $NaClO_4$[5] or 23.7 mg. NaCl or 61 mg. NaI
45 minutes: I^{131} + 50 mg. $NaClO_4$ or 23.8 mg. NaCl or 61 mg. NaI
103–135 minutes: Sacrifice

When the rats were sacrificed 4–4½ hours after radioiodide administration, the schedule was the following:

0 minute : 6 mg. PTU + 50 mg. $NaClO_4$ or 23.7 mg. NaCl or 61 mg. NaI
45 minutes: I^{131} + 25 mg. $NaClO_4$ or 11.9 mg. NaCl or 30.5 mg. NaI
165 minutes: 25 mg. $NaClO_4$ or 11.9 mg. NaCl or 30.5 mg. NaI
285–315 minutes: Sacrifice.

In experiment XVI the 24 rats received a total of 52 mg. of PTU according to the following schedule:

0 hour : 20 mg. PTU suspension
24 hours: 6 mg. PTU solution
32 hours: 20 mg. PTU suspension
44 hours: 6 mg. PTU solution
48–48½ hours: Sacrifice

Sixteen rats were given the radioiodide at 24 hours and 8 rats at 47 hours. Eight rats of the former group and all the animals of the latter received 47.5 mg. of NaCl at 47 hours. The other 8 rats of the first group were injected with 100 mg. of $NaClO_4$ at 47 hours.

3. *Operative procedures*

a) *Nephrectomy.* Bilateral nephrectomy was performed through a single dorsal incision. The rats were anesthetized with nembutal. In experiment XV nephrectomy was carried out transperitoneally since it was combined with ligation of the cardia and the pylorus.

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[5] When a total dose of 200 or 400 mg. of $NaClO_4$ was given, the single doses were proportionately higher.

TABLE 1. THYROID:BLOOD RADIOIODIDE CONCENTRATION (T/B) RATIOS, AS AFFECTED BY PERCHLORATE. CORRELATION WITH THYROID STRUCTURE

Group	Iodide administered	Hours after I^{131}	T/B ratio		% of total gland volume			Gland weight, mg.
			Without ClO_4^-	With ClO_4^{-1}	I^{131} Space	Stroma	Stroma + cells	
Intact	tracer I^{131}	1–1½	(6) 22.7 ±1.9[5]	(12) 0.45 ±0.025	30	(10) 10.2 ±0.72	85.2 ±0.45	(18) 8.3 ±0.35
Hypex[2]	tracer I^{131}	1–1½	(6) 2.5 ±0.72	(16) 0.47 ±0.018	31	(10) 8.6 ±0.53	62.0 ±2.3	(22) 8.2 ±0.33
Hypex + PTU + TSH[3]	tracer I^{131}	1–1½	(8) 83.6 ±9.2	(20) 0.44 ±0.022	29	(10) 10.4 ±0.72	75.4 ±1.7	(28) 8.4 ±0.27
PTU + triiodo-[4] thyronine	tracer I^{131}	1–1½	(6) 56.5 ±4.4	(14) 0.43 ±0.015	29	(10) 12.1 ±0.46	83.7 ±1.3	(20) 22.1 ±1.1
Intact	tracer I^{131}	4–4½	—	(11) 0.41 ±0.029	28	—	—	(11) 7.9 ±0.25
Intact	tracer I^{131} 45 min. after 100 mg. of NaI^{127}	1–1½	—	(8) 0.44 ±0.017	29	—	—	(8) 9.7 ±1.1

In parentheses: number of rats in group used for determinations.

[1] 100–400 mg. $NaClO_4$.

[2] Nine days after hypophysectomy.

[3] Nine days after hypophysectomy. Twenty mg. of propylthiouracil in suspension form and 0.9 U.S.P. unit of TSH injected 24 hours before I^{131} administration.

[4] Twenty mg. of propylthiouracil in suspension form injected daily for 14 days; thereafter 30 μg. of l-triiodothyronine daily for 3 days. Animals killed 24 hours after the last injection.

[5] Mean ± standard error.

b) *Cardiac and pyloric ligation* was performed under nembutal anesthesia in such rats as were subsequently used for the determination of gastric juice-serum radioiodide gradients. The operation was followed immediately by the injection of PTU (see schedules).

c) *Intestinal ligation and radioiodide injection.* In order to study the absorption of radioiodide from the small intestine, the following procedure was carried out. Rats which had received 100 mg. of $NaClO_4$ or 47.5 mg. of NaCl one hour earlier were laparotomized under nembutal anesthesia and ligatures were applied to the pylorus and the cecal end of the ileum. Radioiodide was injected into the duodenum through a gauge 27 hypodermic needle. Thereafter the intestine was placed back into the abdominal cavity and the skin incision was closed with surgical clips. When the rats were sacrificed 30 minutes later, the small intestine was removed *in toto* and dissected free of its mesentery.

4. *Methods used in securing samples of body fluids and tissues and in determining their radioactivity*

Blood was obtained from the abdominal aorta. Some organ-serum radioiodide concentration (O/S) ratios were determined by the method of VanderLaan and Greer (1950). Tissue samples not weighing over 50 mg. were squashed on filter paper under Scotch tape and 0.1 ml. of serum or a 1:10 dilution thereof was pipetted onto filter paper, dried and covered with Scotch tape. The radioactivity of the tissue and serum samples was determined by means of a thin mica end-window Geiger-Müller (beta) counter. The O/S ratio was expressed as the quotient of the activity of 100 mg. of wet tissue over that of 0.1 ml. of serum. This method was used for thyroid, salivary glands, pituitary, adrenals and diaphragm. For thyroid-blood radioiodide concentration ratio determinations 0.1 ml. of blood was laked in 0.9 ml. of distilled water and 0.1 ml. of the hemolysate was used in the manner outlined for serum. In the case of larger organs (kidney, lung, skin, liver, testis, gastrocnemius muscle and stomach wall) either the whole organ or a piece not weighing over 2 gm. was put into plastic tubes, serum samples were collected in similar tubes, radioactivities were determined using a well-type gamma counter (Texas Company), and O/S ratios were computed. Measured stomach juice samples were obtained from the supernatant of centrifuged gastric contents and gamma-counted in plastic tubes. Erythrocyte-plasma radioiodide concentration ratios were determined by collecting blood from rats injected intraperitoneally with 400 U.S.P. units of heparin 20 minutes before exsanguination. The blood thus obtained was centrifuged in plastic tubes at 2800 r.p.m. for 1 hour and then stored in a deep freeze unit for 24 hours. Pieces containing only packed erythrocytes and plasma, respectively, were broken off the two ends of the frozen column of centrifuged blood after its removal from the tube. These pieces were placed into other tubes, weighed, thawed out and counted in the gamma counter. For whole blood-serum radioiodide concentration ratio determinations the blood sample was obtained either by withdrawal into a heparinized syringe or by laking an aliquot of blood in distilled water. Serum was obtained from the same animals by severing the aorta, collecting the extravasated blood and centrifuging it in glass tubes. If heparinized blood was used, the radioactivity of known volumes of both blood and serum was determined by gamma-counting. If laked non-heparinized blood was utilized, measured blood and serum samples were subjected to beta counting. For plasma-serum radioiodide distribution studies plasma was obtained by centrifuging blood withdrawn with a heparinized syringe and serum by the centrifugation of blood collected from the abdominal cavity of the same animals after severance of the aorta. Beta counting of measured samples was used for radioactivity determinations. The amount of radioiodide absorbed from the lumen of the small intestine was computed by comparing the activity of the

intraintestinally injected tracer with that of the dissected small intestine after the half-hour *in vivo* sojourn of the tracer in its lumen. The gamma counter was used in this experiment.

5. *Radioiodide space determinations*

The formula used for the computation of the radioiodide space of various organs (expressed as % of their total volume) was based on the assumption that the radiohalogen is confined to the extracellular phase. This was done in order to facilitate comparison with similarly computed organ radioiodide and radiobromide spaces reported by others (Leblond, 1942, Perlman *et al.*, 1941).

When O/S ratios were determined, the radioiodide space of the organs was expressed as

$$\frac{\text{organ radioiodide concentration} \times 0.93 \times 0.95 \times 100}{\text{serum radioiodide concentration} \times 0.99},$$

where 0.93 and 0.99 are the correction factors for the solid contents of serum and tissue fluid, respectively, and 0.95 is the factor for the Gibbs-Donnan equilibrium. When the radioiodide concentration of whole blood was measured instead of that of serum (Table 1), the above formula was modified in accordance with the observed blood-serum radioiodide concentration ratios (Table 2) and the thyroid radioiodide space was therefore expressed as

$$\frac{\text{thyroid radioiodide concentration} \times 0.93 \times 0.95 \times 0.75 \times 100}{\text{blood radioiodide concentrations} \times 0.99}.$$

TABLE 2. DISTRIBUTION OF RADIOIODIDE IN BLOOD (1–1½ HOURS AFTER SUBCUTANEOUS INJECTION)

Radioiodide concentration ratio	$NaClO_4$ (100 mg.)	NaCl (47.5 mg.)	0
Erythrocytes/plasma	(10) 0.59 ± 0.016	(10) 0.59 ± 0.028	(9) 0.55 ± 0.013
Plasma/serum	(9) 0.95 ± 0.096	(10) 0.98 ± 0.976	—
Whole blood/serum			
a) Heparinized blood	(28) 0.75 ± 0.014	(29) 0.76 ± 0.013	(9) 0.78 ± 0.022
b) Non-heparinized blood	—	—	(20) 0.80 ± 0.010

Number of rats in parentheses. Means ± standard errors.

The body radioiodide space was defined as the volume in which radioiodide would be distributed if its concentration throughout the body were the same as in serum. In expressing this volume as % of body weight the difference between the densities of serum and whole body was neglected.

6. *Histological methods*

Thyroids were fixed in Susa, embedded in paraffin, cut at 6 μ and stained with periodic acid-Schiff-trichrome. The proportion of the various components of the gland was determined on projected slides by the linear measurement technique of Uotila and Kallas (1952), using sections taken at three different levels from each gland.

7. *Statistical analysis of results*

Differences between the means of measurements obtained in comparable groups were analyzed with the aid of Student's "t" test and are called significant in the text if $P < 0.01$.

RESULTS AND DISCUSSION

1. *The radioiodide space of perchlorate-blocked thyroids*

The experiments summarized in Table 1 showed that the radioiodide space of perchlorate-blocked thyroids a) was approximately three times as great as the histologically determined stromal compartment of the gland; b) did not vary in size when alterations of the level of thyrotrophic stimulation caused enlargement of the thyroid or changes in the percentage of thyroid volume occupied by stroma plus parenchymal cells and, on the functional side, profound variations in the activity of the iodide "trap"; c) was not affected by the administration of a large dose of stable iodide before the injection of the tracer, and d) was as great 1–1½ hours after the injection of radioiodide as it was at 4–4½ hours.

Comment. Circumstantial evidence for the anatomical location of the iodide "trap" could only be expected from the determination of the radioiodide space of perchlorate-blocked thyroids (which iodide presumably enters by diffusion only), if the interface across which iodide is actively transported were impenetrable to diffusing iodide. If this premise were fulfilled, and a) the iodide pump were located along the thyroid cell-stromal boundary, the radioiodide space after perchlorate block should be no greater than the stromal compartment of the thyroid; b) if the site of the pump were the thyroid cell-colloid interface, the radioiodide space of the perchlorate-blocked gland should vary parallel with changes in the percentage of thyroid volume occupied by stroma plus parenchymal cells. Actually, the thyroidal radioiodide space after perchlorate treatment was three times as great as the stromal space. Such an extension of the radioiodide space beyond the stromal compartment could hardly have been stimulated by adsorption of radioiodide to structures contained in or bounding the stroma, since the magnitude of the space was not affected by a large dose of stable iodide. Stable iodide would have competed with radioiodide for loci of binding, and thereby would have diminished any apparent enlargement of the radioiodide space due to adsorption of the tracer. Further, the radioiodide space of perchlorate-blocked thyroids was remarkably constant in our experimental groups, although the magnitude of the thyroidal "stroma plus parenchymal cells" compartment was significantly enhanced by thyrotrophin (TSH) and diminished by its lack. Thus there was no indication that iodide diffuses up to but not across the thyroid cell-colloid interface, and there was evidence that the basal portion of the thyroid cell membrane is permeable to diffusing iodide. Our experiments could therefore provide no evidence for the anatomical site of the thyroidal iodide pump. As regards the second objective of our investigations, our results gave no indication that TSH promotes the diffusion of iodide into the thyroid parenchyma.

The constancy of the radioiodide space of perchlorate-blocked thyroids

under a variety of experimental conditions may indicate that in such glands iodide diffuses throughout the stroma and parenchyma, including the colloid. If such is the case, an explanation must be sought for the fact that the thyroidal radioiodide space is only a fraction of the space occupied by the total water of the gland. Thyroidal water content, as determined by desiccation, was found to average 78% of the gland's weight. So far as the thyroid is concerned, equilibration of radioiodide must have been complete by 1–1½ hours after the administration of the tracer, since no increase in the thyroid-blood radioiodide concentration ratio was observed when the rats were sacrificed 4–4½ hours after the injection. Two factors that could hamper the diffusion of radioiodide into the thyroid are a) the electric potential of the thyroid cell membrane and b) complexing of radioiodide with plasma proteins. We are not aware of any information concerning the properties of the thyroid cell membrane. The distribution of radioiodide among the components of blood was the subject of the second series of experiments included in this study.

2. *The distribution of radioiodide in blood (Table 2)*

The erythrocyte-plasma radioiodide concentration (RBC/Pl) ratio was lower than that observed by Boatman and Moses (1951) in rats and by Rall *et al.* (1950) in human blood *in vitro*. The plasma-serum radioiodide concentration ratio was probably not significantly different from 1. The whole blood-serum radioiodide concentration (B/S) ratio can be determined from the hematocrit (H) and the RBC/Pl ratio by the following formula:

$$B/S = \frac{RBC/Pl \times H + 100 - H}{100}.$$

In our experience the hematocrit values of rats (pooled data for animals which received 100 mg. of $NaClO_4$, 47.5 mg. of NaCl or no salt) ranged from 42 to 52 and averaged 48. With the hematocrit at 48 and the RBC/Pl ratio at 0.57, the computed B/S ratio is 0.79. This is in good agreement with the values which we obtained by actual determination of this ratio.

The distribution of radioiodide in blood did not appear to be significantly affected by treatment of the rats with 100 mg. of $NaClO_4$ or equimolecular amounts of NaCl, nor were the B/S ratios obtained by using *in vitro* heparinized blood different from those determined without the use of heparin.

Comment. The reliability of our direct measurement of the RBC/Pl ratio was supported by the agreement between the B/S ratio computed on its basis and that determined experimentally. If the erythrocyte water content is 65% and that of plasma 93% (Rall *et al.*, 1950), and if radioiodide distributed itself evenly throughout both plasma and erythrocyte water, without the occurrence of adsorption or binding, the RBC/Pl ratio should be 0.70. In our experiments it was only approximately 80% of this

value. This may have been due to a) electrolytic dissociation of hemoglobin or b) binding of radioiodide by plasma proteins. The lack or difference between plasma and serum radioiodide concentrations militates against a quantitatively important binding of radioiodide to fibrinogen. Complexing of radioiodide with other plasma proteins may occur and may be the only reason for the apparent disparity between the radioiodide space and the water space of the erythrocyte.[6] However, it cannot fully account for the much greater difference between these spaces in the perchlorate-blocked thyroid and in other organs which will be subsequently discussed.

3. *A survey of the radioiodide space of various organs*

We examined the organ-serum radioiodide concentration (O/S) ratio of a variety of organs and computed their radioiodide space on the basis of these measurements (Table 3). With the exception of the stomach wall

TABLE 3. ORGAN-SERUM RADIOIODIDE CONCENTRATION (O/S) RATIOS AND ORGAN RADIOIODIDE SPACES (AS % OF VOLUME) 1½ HOURS AFTER THE ADMINISTRATION OF I^{131}. EFFECT OF PERCHLORATE

Organ	Expt. No.	Treatment					
		47.5 mg. NaCl		0		100 mg. $NaClO_4$	
		O/S ratio	I^{131} Space	O/S ratio	I^{131} Space	O/S ratio	I^{131} Space
Kidney	II	(10) 0.66 ± 0.024	58%	—	—	(8) 0.70 ± 0.035	61%
Lung	III	(6) 0.60 ± 0.018	53%	—	—	(6) 0.62 ± 0.018	54%
Skin	III	(5) 0.53 ± 0.027	47%	—	—	(6) 0.54 ± 0.014	48%
Submaxillary gland	VI	(9) 0.41 ± 0.022	36%	—	—	(8) 0.42 ± 0.030	37%
Submaxillary gland	XVIII	—	—	(11) 0.39 ± 0.014	34%	—	—
Parotid	XVII	(6) 0.39 ± 0.026	34%	—	—	(6) 0.33 ± 0.023	29%
Spleen	III	(5) 0.38 ± 0.013	33%	—	—	(6) 0.34 ± 0.010	30%
Liver	II	(10) 0.38 ± 0.013	33%	—	—	(8) 0.36 ± 0.018	32%
Liver	XIX	—	—	(7) 0.34 ± 0.011	30%	—	—
Thyroid	I	—	—	—	—	(10) 0.37 ± 0.016	33%
Pituitary	VI	(9) 0.32 ± 0.014	28%	—	—	(10) 0.31 ± 0.012	27%
Pituitary	XIX	—	—	(11) 0.30 ± 0.008	26%	—	—
Adrenal	VI	(10) 0.29 ± 0.021	25%	—	—	(10) 0.29 ± 0.012	25%
Adrenal	XIX	—	—	(11) 0.29 ± 0.012	25%	—	—
Testis	III	(5) 0.22 ± 0.007	19%	—	—	(6) 0.23 ± 0.008	20%
Diaphragm	VI	(10) 0.20 ± 0.011	18%	—	—	(10) 0.20 ± 0.012	18%
Diaphragm	XIX	—	—	(11) 0.15 ± 0.008	13%	—	—

Number of rats per group in parentheses. Means ± standard errors. Experiments designated by the same Roman numeral were carried out simultaneously.

(Table 6), which will be discussed later, and, of course, the non-blocked thyroid (Table 1), the O/S ratios were less than 1 and were not significantly affected by 100 mg. of $NaClO_4$ if the determinations were made 1–1½ hours after the administration of the tracer. The O/S ratios of rats given no chloride were not significantly different from those of rats which received 47.5 mg. of NaCl, with the exception of the diaphragm-serum ratio, which was lower if the animals were not given salt. The radioiodide space of the perchlorate-blocked thyroid was lower than that of kidney, lung and skin, not reliably different from that of salivary glands, spleen

[6] Attempts to show whether some serum radioiodide was $ZnSO_4$-precipitable because of protein-binding were inconclusive, since addition of $Zn(OH)_2$-$BaSO_4$ to a solution of radioiodide in distilled water also resulted in absorption of varying amounts of radioiodide to the precipitate.

and liver, and higher than that of pituitary, adrenal, testis and skeletal muscle.

Comment. The O/S ratios listed in Table 3 do not differ markedly from those obtained for some of these organs by Wallace and Brodie (1937) with stable iodide in dogs and by Leblond (1942) with radioiodide in rats. Wallace and Brodie considered the iodide space to be coextensive with the extracellular space. Leblond, on the other hand, believed that radioiodide does enter cells.

Although we made no quantitative determinations of the stromal compartments of organs other than the thyroid, mere inspection of histologic preparations suggest that the radioiodide space is considerably greater than the extracellular space in all glands that were examined, with the possible exception of the testis. It would be hazardous to attempt a histologic estimation of the extracellular compartment in lung, spleen and skin. In the lung the extremely rich capillary network is in all probability largely responsible for the high O/S ratio. The skin used in our experiments was taken from the anterior aspect of the neck and included the hairs but not the subcutis. Although hair is known to concentrate iodide (Leblond, 1954), this process must be slow, since plucked hair showed negligible radioactivity 1–1½ hours after the administration of radioiodide. The comparatively high O/S ratio of skin samples which consisted of the dense fibrous dermis, the epidermis and its appendages suggests that radioiodide in this organ is not confined to the vascular and tissue fluid compartments, but may penetrate into collagenous fibers, as does chloride (Manery, 1954). The fact that the kidney showed a high O/S ratio is not surprising, since radioiodide-containing urine is concentrated in its nephra. The probable uptake of radioiodide by renal tubular cells will be discussed later. The radioiodide space of striated muscle is probably somewhat larger than its chloride space (cf. Woodbury, 1954). Penetration of some radioiodide into the muscle fibers therefore appears likely.

Of special interest is the fact that salivary glands showed no evidence of an iodide concentrating mechanism similar to that of the stomach (cf. Tables 3 and 6). The existence of concentrated iodide in human saliva as well as gastric juice is well established (Honour *et al.*, 1952). Freinkel and Ingbar (1953) have shown that the human salivary iodide concentrating mechanism is inhibited by thiocyanate. A considerable portion of the combined gastric-salivary radioiodide in rats is contributed by the saliva (Brown, 1955). It remains to be explained, therefore, why the rat's salivary glands failed to contain concentrated radioiodide under the conditions of our experiments, even though the gastric wall did. Johnson and Albert (1951) have also reported a radioiodide concentration of "salivary gland" (submaxillary according to a personal communication of Dr. Albert) lower than that of blood. The ineffectiveness of perchlorate on salivary gland-serum radioiodide concentration ratios is also in striking contrast with its

marked depressing effect on the gastric wall-serum ratio (Table 6). It is, of course, possible that the salivary iodide concentrating mechanism transports iodide across the apical boundary of the cells into the lumen, and that the cells themselves contain no concentrated iodide, but this may hold true for the gastric iodide pump as well. In the likely event of stagnation of saliva in the extensive duct system the iodide content of saliva should influence the salivary gland-serum iodide concentration ratio. Therefore, if a salivary iodide pump similar to that of the stomach existed in rats, one would expect a) a salivary gland-serum iodide concentration ratio higher than that observed and b) a marked influence of perchlorate on this ratio.

It is interesting to compare the radiobromide space (Perlman *et al.*, 1941) of various organs with their radioiodide space. The radiobromide space of rat muscle was found to be 18%, which agrees with the radioiodide

TABLE 4. EFFECT OF PERCHLORATE ON THE BODY RADIOIODIDE SPACE OF NEPHRECTOMIZED RATS

Hours after nephrectomy	Hours after I^{131}	Radioiodide space, % of body weight	
		100 mg. $NaClO_4$	47.5 mg. NaCl
2–2½	1–1½	(5) 25.0 ± 0.9	(6) 34.5 ± 2.0
5–5½	1–1½	—	(3) 38.3 ± 1.3
5–5½	4–4½	(6) 36.2 ± 0.4	(5) 74.1 ± 4.7

Number of rats per group in parentheses. Means ± standard error.

space, but that of the liver was only 22% and that of the adrenal as high as 36%. Such discrepancies between the radioiodide and radiobromide spaces are not easily explained if one assumes that both radiohalogens are confined to the extracellular phase of these organs.

In conclusion, radioiodide appears to diffuse into the cells of at least some organs beside the thyroid. The radioiodide space of all organs examined (with the exception of the thyroid and the stomach wall of animals that had not received perchlorate) appeared to be less than their total water space. The electric properties of the plasma membranes of different cells may play a role in determining the characteristic magnitude of the radioiodide space of different organs.

4. *Effect of perchlorate on the body radioiodide space and the gastric iodide concentrating mechanism*

Having investigated the radioiodide space of various organs, we became interested in the radioiodide space of the whole body, and the effects of perchlorate thereupon. Table 4 shows that perchlorate significantly depressed the body radioiodide space of nephrectomized rats. In both the perchlorate-treated rats and the chloride-treated controls the magnitude of the body radioiodide space increased between 1 and 4 hours after the

administration of the tracer. However, this expansion of the space was much more marked in the chloride-treated animals.

In attempting to explain these observations, we compared organ-serum radioiodide concentration ratios determined 1–1½ hours and 4–4½ hours after the injection of I^{131}. Special attention was paid to organs which are large enough to influence the body iodide space, viz. liver, muscles and skin. Both nephrectomized and unoperated rats were used (Table 5). Neither the difference between the body radioiodide space of perchlorate-treated and chloride-treated rats nor the expansion of this space with time was explained by the findings summarized in Table 5. The organ-serum radioiodide concentration ratios for muscle, skin, submaxillary gland and liver may have been slightly higher 4–4½ hours than 1–1½ hours after the injection of I^{131} in the chloride-treated group, but no such difference was

TABLE 5. ORGAN-SERUM RADIOIODIDE CONCENTRATION (O/S) RATIOS 1–1½, 4–4½ AND 24–24½ HOURS AFTER ADMINISTRATION OF THE TRACER. EFFECT OF PERCHLORATE OR STABLE IODIDE

Organ	Dose of PTU, mg.	Expt. no.	Hours after nephrectomy	Hours after I^{131}	O/S ratio		
					47.5 mg. NaCl	100 mg. $NaClO_4$	122 mg. NaI
Skeletal muscle:							
diaphragm	6	VI	—	1– 1½	(10) 0.20±0.011	(10) 0.20±0.012	—
gastrocnemius	6	VIII	—	4– 4½	(6) 0.23±0.021	(6) 0.19±0.020	—
gastrocnemius	6	IX	—	4– 4½	(9) 0.23±0.013	—	(9) 0.19±0.008
gastrocnemius	6	V	5–5½	4– 4½	(6) 0.18±0.006	(6) 0.20±0.009	—
Skin	6	III	—	1– 1½	(5) 0.53±0.027	(6) 0.54±0.014	—
Skin	6	VIII	—	4– 4½	(6) 0.65±0.046	(6) 0.56±0.028	—
Skin	6	V	5–5½	4– 4½	(6) 0.59±0.016	(6) 0.53±0.012	—
Submaxillary gland	6	VI	—	1– 1½	(9) 0.41±0.022	(8) 0.42±0.030	—
Submaxillary gland	6	VII	—	4– 4½	(5) 0.48±0.040	(7) 0.39±0.032	—
Adrenal	6	VI	—	1– 1½	(10) 0.29±0.021	(10) 0.29±0.012	—
Adrenal	6	VII	—	4– 4½	(7) 0.29±0.021	(7) 0.26±0.010	—
Liver	6	II	—	1– 1½	(10) 0.38±0.013	(8) 0.36±0.018	—
Liver	6	VIII	—	4– 4½	(6) 0.42±0.018	(6) 0.34±0.015	—
Liver	6	V	5–5½	4– 4½	(6) 0.37±0.018	(6) 0.32±0.006	—
Liver	6	IX	—	4– 4½	(10) 0.47±0.022	—	(10) 0.34±0.018
Liver	52	XVI	—	24–24½	(8) 0.44±0.020	(8) 0.33±0.031	—
Liver	52	XVI	—	1– 1½	(8) 0.32±0.010	—	—

Number of rats per group in parentheses. Means ± standard errors. Experiments designated by the same Roman numeral were carried out simultaneously.

observed in the perchlorate-treated group. Further, any depressing effect that perchlorate may have had on organ-serum radioiodide concentration ratios was minimal in comparison with its influence on the radioiodide space of the whole body.

Subsequent investigations showed that the effect of perchlorate on body radioiodide space is probably largely due to inhibition of the gastric iodide concentrating mechanism. Table 6 shows that the gastric wall-serum radioiodide gradient, which was somewhat greater than 1 and did not increase with time, was greatly depressed by perchlorate. More significantly, the gastric juice-serum radioiodide concentration ratio, which was above 15, was reduced to less than 1 by perchlorate. The gastric juice-serum radioiodide gradient was lower in nephrectomized rats, and was markedly greater in such animals when determined 4–4½ hours after I^{131} injection than at 1–1½ hours.

Comment. The shape of the time curve of the body radioiodide space in chloride-treated nephrectomized rats suggests that equilibration of the radioisotope may not have been complete 4 hours after its injection. Myant *et al.* (1950) have also found that the radioiodide space of the human body expands up to 5 hours after administration of the tracer. The body radioiodide space of man, however, was substantially lower (approximately 36% of body weight) than that of rats after 4 hours of equilibration of I^{131}. The body stable iodide space of dogs (Wallace and Brodie, 1937) was also in the vicinity of 40% of body weight. Our findings regarding the large size of the body radioiodide space of nephrectomized rats are, however, in good agreement with Ingbar's (1953) estimate of this space in intact rats. He found that 2–8 hours after the administration of a tracer dose of radioiodide it was distributed in a volume equaling that of the entire body.

Since we could confirm earlier observations (Leblond, 1942 and others) regarding the concentration of radioiodide in the stomach wall and, especially, in the gastric contents, we concluded that the gastric stores of concentrated radioiodide must greatly expand the body radioiodide space

TABLE 6. EFFECT OF PERCHLORATE ON THE STOMACH WALL-SERUM AND GASTRIC JUICE-SERUM RADIOIODIDE CONCENTRATION RATIOS

	Expt. no.	Hours after nephrectomy	Hours after I^{131}	Treatment	
				47.5 mg. NaCl	100 mg. $NaClO_4$
Stomach wall/serum	II	—	1–1½	(10) 1.45±0.10	(8) 0.36±0.020
Stomach wall/serum	XIII	—	4–4½	(5) 1.44±0.26	—
Gastric juice/serum	XIV	—	4–4½	(8) 15.8±2.0	(7) 0.75±0.050
Gastric juice/serum	XV	5–5½	1–1½	(5) 1.9±0.4	—
Gastric juice/serum	XV	5–5½	4–4½	(3) 5.8±0.8	—

Number of rats per group in parentheses. Means ± standard errors.

Inhibition of the gastric iodide concentrating mechanism was previously achieved by means of thiocyanate (Mason, 1952). Perchlorate was now shown to have the same effect. The reduction of the body radioiodide space after perchlorate treatment was therefore easily explained.

The observed increase with time in the body radioiodide space of chloride-treated nephrectomized rats became understandable when we demonstrated that the gastric juice-serum radioiodide gradient increased *pari passu* in such animals (Table 6).

The observation that the gastric juice-serum radioiodide concentration ratio was greater in intact than in nephrectomized rats (Table 6) may be tentatively interpreted by assuming that the kinetics of gastric radioiodide concentrating are essentially similar to those of thyroidal radioiodide "trapping." Wollman (1954) has pointed out that the thyroid-serum radioiodide gradient will rise if the rate of excretion of radioiodide is great relative to that of its equilibration. Gastric radioiodide concentration seems to have a rate of equilibration which is slower than that of thyroidal radioiodide "trapping." Hence, if the gastric and thyroidal iodide pumps are

comparable, stomach juice-serum radioiodide gradients should be even more readily influenced by changes in the rate of radioiodide excretion than are T/S ratios. One would therefore expect a markedly higher gastric juice-serum radioiodide gradient in animals with intact kidneys than in nephrectomized animals.

The body radioiodide space of perchlorate-treated nephrectomized rats (whose gastric iodide pump was presumably nonfunctional) significantly increased with time. This could not be ascribed to an expansion of the radioiodide space of any of the organs examined. It is possible that there was a slow diffusion of radioiodide into some organ system that was not investigated, e.g. the skeleton and the central nervous system. The body radioiodide space of 36% which was found 4–4½ hours after the injection of I^{131} in perchlorate-treated rats exceeds the extracellular space estimated in other species by any of the conventional methods (Elkington and Danowski, 1955). This is in agreement with the suggestion that radioiodide is not confined to the extracellular phase, even when the iodide pumps of the body are blocked.

5. *Effect of perchlorate and stable iodide on hepatic radioiodide uptake*

Leblond (1942) found less radioiodide in the livers of rats after pretreatment with large doses of stable iodide than if no stable iodide was administered. He suggested that the liver possesses a mechanism for the accumulation of iodine which can be saturated. Our findings confirmed and extended these observations (Table 5). The liver-serum radioiodide concentration (L/S) ratio was not significantly altered by perchlorate-pretreatment if the determination was made 1–1½ hours after the injection of I^{131}. Four to 4½ hours after the administration of the tracer, however, the L/S ratio was significantly lower in perchlorate-treated rats than in chloride-treated controls. Stable iodide in amounts equimolecular to those of perchlorate had the same effect. The L/S ratio had not increased beyond the 4–4½ hour value when it was determined 24–24½ hours after radioiodide administration (1–1½ hours after the injection of NaCl). The 24–24½ hour L/S ratio was significantly lowered when $NaClO_4$ was injected instead of NaCl 1–1½ hours before the rats were killed. The rats used for the determination of the 24–24½ hour L/S ratio had received 52 mg. of propylthiouracil (PTU) instead of the customary 6 mg. In order to examine the possible effect of this pretreatment *per se* on hepatic radioiodide uptake, the 1–1½ hour L/S ratio was also examined in such animals. It was significantly lower than the 24–24½ hour ratio of rats which had not received perchlorate and was not reliably different from the L/S ratios found at any time interval after I^{131} administration in perchlorate-treated rats.

Comment. Our findings indicate that not all of the hepatic radioiodide enters the organ by simple diffusion. Rather, there appears to exist another hepatic iodide space in addition to the iodide diffusion space of the organ.

The entry of radioiodide into this second compartment occurs slowly and can be inhibited with stable iodide as well as perchlorate. Furthermore, radioiodide can be discharged from this compartment by means of perchlorate. Lastly, once radioiodide has entered the compartment, it seems to equilibrate with serum iodide, and no further increase in the L/S ratio occurs with time. Such an increase would be expected if radioiodide became bound within the liver instead of remaining freely exchangeable. Our results are compatible with the idea that the liver possesses an iodide pump essentially comparable with that of the thyroid, except that the equilibration of pumped hepatic iodide with serum iodide is slower than in the case of the thyroid, and that the hepatic mechanism is probably much less efficient than its thyroidal counterpart, since the thyroid-serum radioiodide gradient may reach 400, whereas the liver-serum radioiodide concentration does not exceed 0.5.

6. *Effect of perchlorate on the intestinal absorption and the excretion of radioiodide*

Table 6 shows that even after the administration of a dose of perchlorate which presumably blocked the gastric iodide pump as completely as it blocked that of the thyroid, radioiodide entered the gastric juice. Since the epithelium of the stomach is interposed between the blood vessels of the stomach wall and the gastric lumen, diffusing radioiodide must have passed through the epithelial cells or the intercellular cement substance. A similar passage of iodide across the intestinal epithelium must occur, since little of the gastric radioiodide can be recovered in the feces (Leblond, 1942). We found that intestinal absorption of radioiodide is not specifically affected by perchlorate and may therefore be due to simple diffusion. In 4 rats which were injected with perchlorate the mean absorption of a dose of radioiodide from the small intestine was 78% within one-half hour. In 3 rats injected with chloride instead of perchlorate an average of 64% of an intraintestinally injected dose of radioiodide was absorbed during the same period.

We have reasons to believe that iodide enters the cells of the renal tubules. The fact that a) renal clearance of iodide is promoted by chloride (Riggs, 1949) and that b) iodide deficient diets are more goitrogenic if they are supplemented with NaCl (Axelrad *et al.*, 1955) is most easily explained by the assumption that chloride competes with iodide for a renal tubular absorptive mechanism. Our experiments suggest that perchlorate is even more effective in this regard. The 4 hour urinary excretion of radioiodide in the study of Johnson and Albert (1951) on rats which received a commercial regimen amounted to approximately 16% of the administered dose. In 6 of our rats which were injected with 47.5% mg. of NaCl the 4 hour loss of radioiodide from the body was $31 \pm 3\%$ (mean $\pm$ standard error) of the dose. This value was arrived at by comparing the activity of

the injected tracer with that of blood samples and taking into account the magnitude of the body radioiodide space as determined in nephrectomized rats (Table 4). Similar measurements and calculations indicated that in 6 rats treated with 100 mg. of $NaClO_4$ $62 \pm 1\%$ of an injected radioiodide tracer was excreted within 4 hours. In view of these results a systematic study of the effect of various anions on the renal clearance of radioiodide is called for.

CONCLUSIONS

The following is a brief synopsis of the distribution of radioiodide in the body of propylthiouracil-treated rats and the effects of perchlorate thereupon.

The thyroid and the stomach possess iodide pumps which can be inhibited by perchlorate. Although iodide concentrations many times those of serum can be found in the thyroid and in gastric juice, the stomach wall-serum iodide concentration ratio is not much over 1. There is no cogent evidence that iodide is concentrated within any cell of the mammalian body. The thyroidal iodide concentrating mechanism may transport iodide across the apical membrane of the thyroid cell into the colloid. The presence of concentrated radioiodide in the follicular lumen has been radioautographically demonstrated by Pitt-Rivers and Trotter (1953) and Doniach and Logothetopoulos (1955). The stagnation of some gastric juice with its highly concentrated iodide in the canaliculi and glandular lumina of the stomach mucosa could fully explain why the iodide concentration of the stomach wall exceeds that of serum.

Curiously, there seems to be no evidence for the existence of a salivary iodide pump in the rat. On the other hand, the liver appears to possess a mechanism for the uptake of iodide which is probably less efficient than that of the thyroid or even the gastric wall but nevertheless may be essentially similar, since it too is inhibited by perchlorate. This hepatic iodide pump (?) may also be concerned with the transfer of iodide into the bile rather than into an intracellular compartment.

There is circumstantial evidence for the existence of a renal tubular mechanism concerned with the active uptake of iodide from the glomerular filtrate. This mechanism seems to be blocked by perchlorate and, although less efficiently, also by chloride, which is not known to affect the iodide pumps of thyroid and stomach.

When the iodide concentrating mechanisms of the body are blocked with perchlorate, iodide probably diffuses through the epithelial lining of the stomach. It is absorbed from the small intestine, presumably also by diffusion through the epithelium. It enters the erythrocyte. The iodide space of several organs, including the perchlorate-blocked thyroid, is clearly larger than their stromal compartment. This suggests that iodide diffuses into a wide variety of cells. In fact, there is no conclusive evidence that iodide remains confined to the extracellular compartment of any organ.

SUMMARY

1. The radioiodide space of rat thyroids, the iodide "trap" of which was completely blocked with sodium perchlorate, was about 30% of the total gland volume, whereas the histologically determined stromal compartment was approximately 10%. This suggests that radioiodide can diffuse into thyroid cells. Thyrotrophin had no effect on the thyroidal radioiodide diffusion space.

2. The radioiodide space of erythrocytes was approximately 80% of their water space. The radioiodide concentration of plasma was not significantly different from that of serum. Perchlorate did not affect the distribution of radioiodide among the components of blood.

3. The radioiodide space of kidney, lung and skin was greater than that of the perchlorate-blocked thyroid, that of salivary glands, spleen and liver about the same, and that of pituitary, adrenal, testis and skeletal muscle smaller. The radioiodide space of these organs (determined 1–1½ hours after the administration of the tracer) was not significantly affected by perchlorate. It is suggested that radioiodide diffuses into the cells of at least some organs other than the thyroid.

4. The body radioiodide space of nephrectomized rats treated with perchlorate increased from 24% of the body weight to 36% between 1 and 4 hours after the administration of I^{131}. In chloride-treated controls the corresponding values were 35% and 74%. The diminution of the radioiodide space in perchlorate-treated animals is believed to be largely due to inhibition of the gastric iodide concentrating mechanism.

5. The liver appears to possess an iodide compartment which equilibrates slowly with serum iodide. The entry of radioiodide into this compartment was prevented by perchlorate or stable iodide. Perchlorate discharged radioiodide from the compartment.

6. Intestinal absorption of radioiodide was at least as fast in perchlorate-treated rats as in chloride-treated controls. Perchlorate appeared more effective than chloride in enhancing the excretion of radioiodide, presumably by interfering with its renal tubular absorption.

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STUDIES OF 17-HYDROXYCORTICOSTEROIDS: ADRENALECTOMY AND HYPOPHYSECTOMY IN GUINEA PIGS AND THEIR EFFECTS ON PLASMA 17-OHCS CONCENTRATIONS[1]

THOMAS A. GOOD,[2] ROBERT S. ELY, LAWRENCE R. HEISELT,[3] ALAN K. DONE,[4] AND VINCENT C. KELLEY[5]

Lockhart Memorial Laboratory, Department of Pediatrics, University of Utah College of Medicine and the Heart Laboratories of the Utah State Rheumatic Fever Clinics, Salt Lake City, Utah

CONSIDERABLE species differences have been observed with regard to the types of hormones secreted by the adrenal cortex (Bush, 1951; Done, Ely, Raile and Kelley, 1952). Among the common small laboratory animals the guinea pig has an adrenal hormone pattern most closely approximating that in man. Therefore, in this laboratory the guinea pig has been the experimental animal employed in studies of adrenal cortical function.

For certain more definitive experiments regarding such function, it is necessary to employ hypophysectomized or adrenalectomized animals. Hypophysectomy and adrenalectomy in the guinea pig have been employed only uncommonly in the past because of the technical difficulty of these procedures. The purpose of this communication is to describe these technics, with certain modifications which make them satisfastory for routine use in the experimental laboratory, and to present certain studies of the effect of these procedures on plasma 17-hydroxycorticosteroid (17-OHCS) concentrations in guinea pigs.

MATERIAL AND METHODS

At the time of obtaining blood samples guinea pigs were rapidly anesthetized in an ethyl ether atmosphere and blood was drawn from the abdominal aortas into heparinized syringes. Plasma samples were separated by centrifugation and stored frozen until used for determination of 17-OHCS by the method of Nelson and Samuels (1952) as modified by Eik-Nes, Nelson and Samuels (1953).

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[2] Post-doctorate Research Fellow, Arthritis and Rheumatism Foundation.

[3] Present address: Department of Pediatrics, University of Oregon Medical School, Portland.

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EXPERIMENTAL

Plasma 17-OHCS in small vs. large intact guinea pigs

Early in the course of these studies it was observed that the plasma 17-OHCS concentrations in intact small guinea pigs were considerably higher than those in large animals. Table 1 compares the values observed in these two groups. In small guinea pigs (150–300 gm.) the mean 17-OHCS concentration was 109 ± 18.5 μg.%. In the larger animals (450–1200 gm.) it was 36 ± 3.5 μg%. Within the latter group there was no apparent correlation between weight of the animal and steroid concentration.

TABLE 1. PLASMA 17-OHCS IN GUINEA PIGS
(Relationship to weight of animals)

Group	Weight (gm.)	No.	Plasma 17-OHCS (μg.%)	
			Mean	S.E.M.
Large	450–1200	25	36	± 3.9
Small	150– 300	8	109	±18.5
			P<0.01	

Technic for adrenalectomy in guinea pigs

To facilitate exposure of the adrenal glands, the guinea pigs are fasted for 12–24 hours before operation; otherwise the distended intestines interfere with visualization of the operative field. The adrenal glands are approached, under sterile technic, by the dorsal route as in the standard adrenalectomy procedure for the rat. A transverse skin incision is made at the level of the costovertebral angles. For removal of the right adrenal gland, the abdominal cavity is entered by an incision, as close to the costal margin as possible, extending anteriorly from the heavy longitudinal back muscles, and just large enough to permit the introduction of a mastoid retractor. The incision is enlarged by opening the mastoid retractor fully, which fixes it in place; this allows upward tension to be exerted on the retractor by a strong rubber band attached to a fixture above the operative field. Such suspension permits the abdominal contents to drop ventrally and provides adequate visualization for removal of the gland. Illumination is provided by a head-lamp.

Anatomically, the right adrenal gland is attached by reflections of the peritoneum posteriorly and superiorly to the diaphragm, anteriorly and superiorly to the liver, and inferiorly to the upper pole of the kidney; medially it is in juxtaposition to the inferior vena cava. With a curved mosquito forceps the adrenal gland and kidney are retracted caudally. The peritoneal reflections to the liver and diaphragm, now under tension, are dissected away with a strabismus scissors, freeing the adrenal down to the vena cava. The gland then is retracted away from the kidney, allowing sharp dissection along the inferior side about two thirds of the distance to the vena cava. The adrenal pedicle is clamped with a small neurosurgical silver clip (3–5 mm.) and the dissection is continued between the clip and gland toward the vena cava. The adrenal gland is now free except for the portion which is contiguous with the wall of the vena cava. Because of this proximity of the gland to the vessel, dissection between the two frequently results in rupture of the vessel wall and exsanguination. The hazards involved at this point are reduced by clamping a large neurosurgical silver clip (6–8 mm.) very tightly at the periphery of the adrenal gland and longitudinally along the vena cava, as previously employed for adrenalectomy in rabbits (Good and Good, unpublished; Good and Thomas, 1954). In some instances this involves impingement upon the wall of the vena cava with narrowing of its lumen. The gland now is removed intact by sharp dissection.

At all times care is taken to dissect as closely to the gland as possible without rupturing the capsule. Because of the delicacy of the procedure it is essential to keep the field clear by controlling the bleeding from small vessels with cotton applicator swabs. The left adrenal gland, which presents less difficulty, is removed in a similar manner except that the last step, using the large clip, is unnecessary.

Effect of adrenalectomy on plasma 17-OHCS

Table 2 shows the effects of adrenalectomy on plasma 17-OHCS concentrations in guinea pigs. In animals weighing 350 to 450 gm. sodium pentobarbital[6] (3 mg./100 gm. body weight) was administered intraperitoneally as the anesthetic agent. Immediately following adrenalectomy each animal was given 20 cc. normal saline hypodermically, and 0.3 cc. of penicillin-streptomycin suspension[7] I.M. Twenty-four hours after adrenalectomy the animals were sacrificed and plasma 17-OHCS concentrations were determined. As indicated in Table 2 these concentrations consistently were zero in the adrenalectomized animals.

TABLE 2. EFFECT OF ADRENALECTOMY AND OF HYPOPHYSECTOMY ON PLASMA 17-OHCS IN GUINEA PIG

Group	Adrenalectomy				Hypophysectomy							
					Large				Small			
	No.	Weight (gm.)	17-OHCS (μg.%)		No.	Weight (gm.)	17-OHCS (μg.%)		No.	Weight (gm.)	17-OHCS (μg.%)	
			Mean	S.E.M.			Mean	S.E.M.			Mean	S.E.M.
Intact Control	25	450–1200	36	±3.5	25	450–1200	36	±3.5	8	150–300	109	±18.5
24 Hours after Operation	5	350– 450	0	±0.0	18	500–1000	8	±1.6	12	150–300	8	± 2.0

Hypophysectomy technic in guinea pigs

Hypophysectomy in the guinea pig, although described in 1933 by McPhail and Parkes, has been a procedure largely abandoned because of its technical difficulties and high mortality rate. These difficulties were circumvented satisfactorily in the early stages of these studies by employing young guinea pigs (150–300 gm.). After considerable experience had been achieved in hypophysectomizing small guinea pigs and certain modifications in the procedure described by McPhail and Parkes (1933) had been introduced, it was possible to use the technic in larger animals with mortality of about 25%.

The parapharyngeal approach described by McPhail and Parkes was used. A tracheotomy tube constructed from a size 18 short-bevel needle, bent in the shape of a U, is inserted into the trachea through a tracheotomy made a distiance of approximately five tracheal rings below the larynx. This facilitates the administration of anesthesia and helps prevent obstruction of the airway during the operation. The vital structures and anterior strap musculature of the neck at the level of the larynx are spread with a blunt-tipped retractor fashioned from a simple curved thumb-forceps. This exposes the deep paired anterior vertebral muscles, which are followed forward to their insertions on the basioccipital and basisphenoid portions of the skull. The posterior superior reflections of the pharyngeal musculature originating on the basisphenoid also can be identified.

[6] Veterinary nembutal solution, Abbott (R), .05 cc./100 gm. body weight.

[7] Containing 200,000 units procaine penicillin and 0.25 gm. streptomycin per cc.

By careful dissection with a periosteal elevator the attachments of these vertebral and pharyngeal muscles to the basisphenoid and basioccipital bones are separated enough to expose the suture line and an area about 5 mm. anterior to it.

This suture line is used as the landmark for trephining. A number 11 dental drill is used to make a burr hole in the midline of the basisphenoid bone, approximately 2 to 5 mm. anterior to the suture line, depending upon the animal's size. With the guinea pig positioned so that its nose is toward the operator, the basisphenoid bone is at an angle downward; this must be remembered and care taken that the hole is drilled perpendicular to the bone surface. If the hole is not centered, or is too far posteriorly, massive hemorrhage is likely to occur because of rupture of the cavernous sinuses which course along each side of, and communicate behind, the pituitary gland. When the drill hole is properly placed the two anterior lobes of the pituitary can be identified and removed by suction. The large posterior lobe and stalk usually are removed at the same time. A layer of dura, penetrated only by the stalk of the pituitary, separates the pituitary from the brain and prevents injury to the latter unless excessive suction is used. Hemorrhage from the trephine hole is stopped with a cotton tipped applicator; it is not necessary to use bone wax. The skin is closed with clips and the tracheotomy tube is left in place.

Mortality from the procedure is caused by hemorrhage, excessive anesthesia, brain injury, or respiratory complications. With careful technic an experienced operator usually will have no difficulty with the first three factors. The most common serious complications of this procedure are respiratory in nature, consisting of either obstruction or infection. The authors feel that one of the causes of high post-operative mortality is the obstruction which results from tearing the posterior pharyngeal musculature. This difficulty usually can be obviated by careful elevation of these muscles, without severing them, in exposing the suture line of the basisphenoid and basioccipital bones. As discussed by McPhail and Parkes (1933), accumulation of secretions in the respiratory tract may produce either obstruction or infection, and accounts for a considerable proportion of the respiratory complications. Tracheotomy reduces this problem considerably. The tracheotomy is made as small as possible eliminating the need to close it after the cannula is removed. The duration of anesthesia is as brief as possible and the tracheotomy cannula is left in place until the animal has recovered.

Effects of hypophysectomy on plasma 17-OHCS

Effects of hypophysectomy on plasma 17-OHCS concentrations in both small and large guinea pigs are shown in Table 2. The animals in this experiment were hypophysectomized rapidly under drop ether anesthesia. The plasma 17-OHCS concentrations 24 hours after hypophysectomy were decreased to low values in both groups. For 18 large animals the mean value was 8 ± 1.6 μg.% and for 12 small animals, 8 ± 2.0 μg.%. In contrast to the uniformly undetectable 17-OHCS values after adrenalectomy, only 4 of 30 animals had zero 17-OHCS values 24 hours after hypophysectomy.

Effect of time interval post-hypophysectomy

In this experiment small guinea pigs, weighing 150–300 gm. were hypophysectomized rapidly under drop ether anesthesia. The animals were sacrificed at various time intervals following hypophysectomy and plasma 17-OHCS concentrations were determined. These data are presented in

TABLE 3. PLASMA 17-OHCS IN GUINEA PIGS AT DIFFERENT TIMES FOLLOWING HYPOPHYSECTOMY

Group	Adequacy of hypophysectomy					
	No "TAG"			"TAG"		
	No.	Plasma 17-OHCS (μg.%)		No.	Plasma 17-OHCS (μg.%)	
		Mean	S.E.M.		Mean	S.E.M.
Control	8	109	±18.5	—	—	—
2 hr. $\bar{p}$ hypox.	12	71	± 9.7	10	111	±17.5
6 hr. $\bar{p}$ hypox.	8	19	± 6.4	8	171	±24.4
12 hr. $\bar{p}$ hypox.	8	14	± 2.9	2	71	—
24 hr. $\bar{p}$ hypox.	12	8	± 2.0	—	—	—
48 hr. $\bar{p}$ hypox.	8	11	± 3.4	5	135	±37.6

Table 3 and illustrated in Figure 1. In animals with complete hypophysectomy the plasma 17-OHCS concentrations decreased rapidly during the first 6 hours after operation and thereafter more slowly, with no essential difference in the values at 12, 24 or 48 hours. In contrast, in the animals with pituitary "tags" remaining at autopsy the plasma 17-OHCS concentrations did not decrease during the 48 hour period following hypophysectomy. The variability in steroid concentration was great in these animals, some of them exhibiting rather extreme elevations of plasma 17-OHCS concentrations, presumably as a reflection of the "stress" of the surgical procedure.

Response of hypophysectomized animals to ACTH

Table 4 shows the plasma 17-OHCS and adrenal ascorbic acid responses to ACTH in small guinea pigs 48 hours after hypophysectomy. In the hypophysectomized controls the mean plasma 17-OHCS concentration was 11 μg.%, and 2 hours after ACTH it was 71 μg.%. Simultaneously the adrenal ascorbic acid concentration decreased from 212 to 82 mg./100 gm. adrenal. Hence, adequate adrenal cortical secretory responses to ACTH stimulation occurred in these animals.

TABLE 4. RESPONSE OF HYPOPHYSECTOMIZED GUINEA PIGS TO ACTH
(48 Hours after hypophysectomy)

Group	No.	Weight (gm.)	Plasma 17-OHCS (μg.%)		Adrenal ascorbic acid† (mg./100 gm. Adrenal)	
			Mean	S.E.M.	Mean	S.E.M.
Hypox. Control	8	150–300	11	±3.4	212	±16.1
+ACTH*	4	150–300	71	±3.6	82	± 9.5
			P = <0.01		P = <0.01	

* 2 hours after 5 I.U. ACTH, I.M.
† Method of Roe, J. H. and Kuether, C. A. (1943).

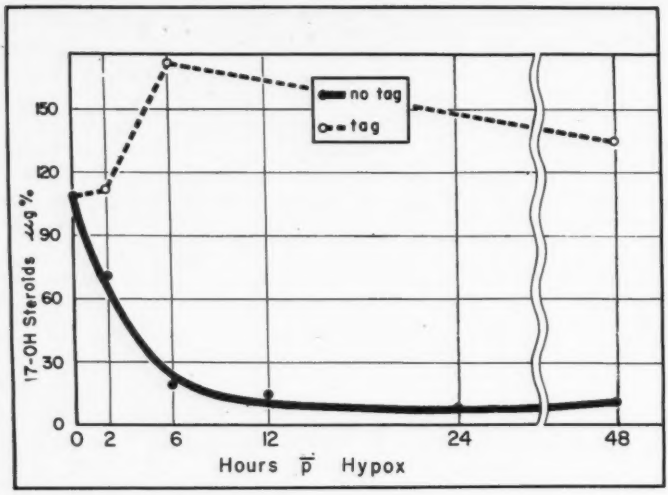

FIG. 1. Comparison of effects of complete and incomplete hypophysectomy on plasma 17-OHCS in guinea pigs.

Effects of anesthesia on plasma 17-OHCS

For certain purposes it is desirable that hypophysectomy be performed using sterile technic. However, since the animal must be anesthetized for purposes of sterile preparation of the skin and draping and since the actual operative procedure requires more time when done under sterile technic, the anesthesia must be prolonged considerably. Table 5 shows the effect of different types of anesthesia on the plasma 17-OHCS concentrations in large guinea pigs 24 hours after hypophysectomy. When prolonged ether anesthesia (45–70 minutes) was used, extreme elevations of plasma 17-OHCS concentrations were found 24 hours after operation. In contrast, when brief ether anesthesia (10–15 minutes) was used, for the more rapid, non-sterile operation, very low 17-OHCS concentrations were found at 24 hours. Also, when sodium pentobarbital was used for prolonged anesthesia (3–4 hours) the 17-OHCS values 24 hours after operation were very low.

TABLE 5. EFFECT OF ANESTHESIA ON PLASMA 17-OHCS 24 HOURS FOLLOWING HYPOPHYSECTOMY

Anesthesia for Hypophysectomy	No.	Plasma 17-OHCS (µg.%)*	
		Mean	S.E.M.
Ether (rapid	18	8	± 1.6
Ether (prolonged)	9	175	±45.6
Pentobarbital	4	7	± 2.3
Controls	25	36	± 3.9

* Values 24 hours after hypophysectomy.

DISCUSSION

The similar pattern of adrenal cortical steroid production by the guinea pig and by man has prompted use of the former for experimental studies of adrenal function. As demonstrated by data presented here the circulating concentration of 17-OHCS in guinea pigs depends upon the size, and presumably the age, of the animals. This must be remembered in evaluating the results of studies concerning adrenal cortical function in these animals.

Interpretation of data obtained in the intact animal concerning adrenal cortical function is subject to certain limitations because of the regulatory role of the anterior hypophysis upon this function. The development of a satisfactory technic for hypophysectomy in the guinea pig permits use of this animal in experiments designed to evaluate such influence. Similarly, a satisfastory technic for adrenalectomy allows further investigation of corticosteroid metabolism in this animal without the interference of continued steroidogenesis.

In adrenalectomized guinea pigs the plasma 17-OHCS concentrations decreased to zero within 24 hours after operation. However, in the hypophysectomized animals, although these concentrations decreased rapidly during the first few hours after surgery, they appeared to stabilize at a low level. This is in conformity with previous observations of De Gurpide (1953) and of Sweat and Farrell (1954) in the hypophysectomized dog. Similar observations have been reported by Macchi and Hechter (1954) in the perfusates of extirpated beef adrenal glands after removal of ACTH from the perfusion medium.

The elevated plasma 17-OHCS concentrations in the animals in which pituitary "tags" were found at autopsy indicate that a small residual amount of anterior hypophyseal tissue is capable of exerting a profound adrenal cortical stimulation; therefore it is important that each animal be autopsied carefully to determine whether a pituitary "tag" is present.

The degree of steroid response to ACTH administered 48 hours after hypophysectomy suggests that such an experimental model may provide a practical method for assaying the steroidogenic activity of ACTH in blood. This is particularly interesting in view of recent reports which indicate, at least under certain circumstances, that a poor correlation exists between the adrenal ascorbic acid depleting and steroidogenic activities of ACTH (Done, Ely, Heiselt and Kelley, 1952; Liddle, Island, Rinfret and Forsham, 1954). Although adrenal ascorbic acid depletion technics for ACTH assay are the best available at the present time (Paris, Upson, Sprague, Salassa and Albert, 1954), a technic directly measuring steroidogenesis would be preferable. The possibility of developing such a technic employing the hypophysectomized guinea pig is being investigated.

The data presented suggest that the type and duration of anesthesia used for hypophysectomy influences profoundly the 17-OHCS concentrations 24 hours after operation. Following brief ether anesthesia as employed for hypophysectomy with non-sterile technic the plasma 17-OHCS

concentrations 24 hours after operation uniformly were low. However, following prolonged ether anesthesia (45–70 minutes), as required for hypophysectomy with sterile technic, these values were grossly elevated. On the other hand following hypophysectomy with sterile technic under prolonged anesthesia with pentobarbital such elevations were not apparent. Since it is known that in the presence of liver damage the half-life of hydrocortisone is lengthened (Brown, Willardson, Samuels and Tyler, 1954), it seems possible that prolonged ether administration produced hepatic damage in these animals sufficient to account for the elevated 17-OHCS concentrations 24 hours after operation whereas no such damage occurred in the animals anesthetized with pentobarbital or exposed only briefly to ether. Unfortunately the livers of the animals were not examined microscopically.

SUMMARY

A detailed technique for adrenalectomy and hypophysectomy in guinea pigs is described and the factors which cause operative failure are discussed. The influence of pituitary or adrenal extirpation on circulating plasma 17-OHCS in small and large guinea pigs is presented.

The plasma 17-OHCS concentrations fall to zero values within twenty-four hours following adrenalectomy. After hypophysectomy a rapid decrease in the plasma 17-OHCS concentration results during the first few hours post-operatively. However, these concentrations do not decrease to zero, but "plateau" at a low level. It was found that prolonged ether anesthesia for the hypophysectomy procedure results in elevated 17-OHCS concentrations twenty-four hours after operation. Brief ether anesthesia or anesthesia with pentobarbital does not cause such elevations.

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VARIATIONS IN SENSITIVITY OF OVARIECTOMIZED GUINEA PIGS TO RELAXIN[1]

MATTHEW W. NOALL[2] AND EDWARD H. FRIEDEN

Arthritis Research Laboratory, the Boston Dispensary and the Cancer Research and Cancer Control Unit, Department of Surgery, Tufts University School of Medicine, Boston, Massachusetts

IN THE course of routine relaxin assays performed in this laboratory during the past two years, it was observed that the guinea pigs which composed the assay colony exhibited reproducible variations in their sensitivity to relaxin. A detailed study of these phenomena and their relationship to reliable assay performance has been made.

METHODS AND RESULTS

The assay procedure generally followed that of Abramowitz *et al.* (1944), as modified by Frieden and Hisaw (1950). Ovariectomized guinea pigs were treated with 1 μg. estradiol 17-β daily for four days. On the fifth day the animals were palpated and those which exhibited significant mobility of the symphysis pubis were dismissed from consideration in the assay. Each guinea pig of a group of 8 to 12 was given a subcutaneous injection of 1 cc. of saline solution of the test material and palpated 6 hours later. The results were expressed in terms of "total response," in the calculation of which the intensity of response of each animal, as well as the percentage of animals of each group which respond, is included (Frieden and Hisaw, 1950). A value of 120 for the total response defines 1 guinea pig unit (GPU). Following the assay, each group was rested for one week before estrogen treatment was initiated preparatory for another assay.

Groups of 20 to 40 young (300–350 gm.) guinea pigs were added to the assay colony one to three weeks after ovariectomy. Each group thereafter constituted an independent subdivision of the assay colony, and remained segregated during its useful life span.

Response Pattern.—If the sensitivity to relaxin of such a group of guinea pigs is tested at intervals during the ensuing six months, a characteristic pattern is observed. The response to the first test dose of relaxin is invariably slight, but increases rapidly on successive assays, generally reaching a maximum at the second or third test dose.[3] This sensitivity is maintained

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[2] Public Health Research Fellow of the National Institute of Arthritis and Metabolic Diseases.

[3] The rapidity with which maximum sensitivity is reached appears to depend upon the length of time which is allowed to elapse between ovariectomy and the first priming dose of estrogen. Thus, maximum sensitivity was observed at the second test dose of relaxin when animals were introduced into the colony one week after ovariectomy. When a lapse of five weeks occurred, however, the appearance of a maximum response was delayed to the fifth or sixth test does. This phenomenon is probably related to the extent of ossification of the symphysis which occurs in the absence of estrogen.

during several subsequent assays, after which a decline in responsiveness sets in. The onset and rate of decline varies considerably between groups. Table 1 lists typical data showing changes in sensitivity of representative groups of animals. Group III was castrated at the end of October, introduced into the colony in early December, and suffered progressive loss in sensitivity beginning sometime between February and April. Groups IV and V ran similar courses in 3 and $4\frac{1}{2}$ months respectively. It should be noted that the loss of sensitivity indicated in Table 1 may not simply be assigned to increased body size. For example, between December and May, Group III approximately doubled in average weight; its sensitivity to relaxin, however, decreased more than 8-fold.

TABLE 1. EFFECT OF REPEATED INJECTIONS UPON SENSITIVITY OF GUINEA PIGS TO RELAXIN PREPARATIONS

Guinea pig group	Preparation	Assay* date	Dose, μg.	Per cent response	Total response
III	MN-4	12/30/54	40	78	125
	MN-4	1/14/55	40	78	125
	123-1	1/28/55	50	72	116
	123-1	4/22/55	100	68	110
	123-1	5/ 6/55	200	56	85
	123-1	6/30/55	200	29	20
IV	123-1	4/28/55	50	94	200
	123-1	5/27/55	50	72	115
	123-1	6/10/55	50	61	73
	123-1	6/16/55	100	50	65
	124-1	6/24/55	100	75	135
	124-1	7/15/55	150	38	30
V	MN-4	3/25/55	40	95	198
	MN-4	4/22/55	40	78	163
	123-1	6/16/55	100	100	220
	123-1	6/30/55	50	72	123
	123-1	7/22/55	100	38	30
	MN-4		100	63	75

* Each group of animals was used for assay at intervals of two weeks. Where greater intervals are indicated, other preparations than those included above were assayed.

The development of a refractory state in one group of assay animals is strikingly shown in Figure 1, in which are plotted dose-response curves for a single relaxin preparation at intervals during a six-month period. It is clear that the loss of sensitivity is associated with relative inability of all but a few animals to respond, no matter what dose is given, i.e., most of the guinea pigs are in a refractory condition rather than in a state of reduced sensitivity.

Similar data were obtained with another group of 31 guinea pigs, this time using a relaxin preparation derived from pregnant rabbit serum. On October 20, 1953, near the end of its useful life, 55%, or 17 of the group were found to relax in response to the administration of 0.25 mg. (1 GPU) of pregnant rabbit serum preparation. One month later the proportion of

those responding to the same dose of the preparation was less than 40%, and no significant increase in response was obtained when the amount injected was increased 4-fold. The refractory state was shown to persist for at least six weeks, even though no estrogen or relaxin was administered in the interim.

Possible Mechanisms of Resistance.—Two possible mechanisms of the loss of sensitivity have been explored. The first was the possibility that the apparent decrease in relaxin sensitivity was in fact due to an increased estrogen requirement, since the response to relaxin depends upon estrogen conditioning of the symphysis. However, no increase in responsiveness could be demonstrated in a refractory group when the amount of estrogen administered was increased twenty-fold.

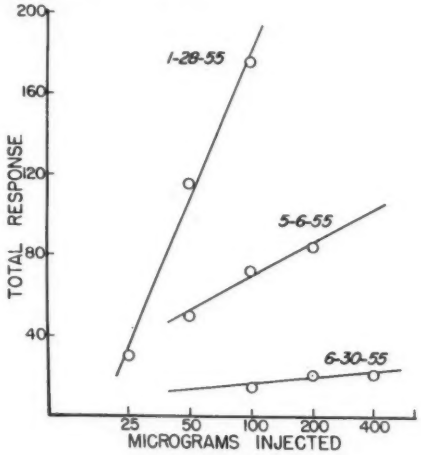

FIG. 1. Response of guinea pigs of group III to relaxin preparation 123-1 at intervals during six months.

A second possible mechanism involved the formation of a specific anti-relaxin antibody in the blood of resistant animals.

Two pools of guinea pig serum were collected, one from relaxin-sensitive pigs and the other from a group of relaxin-resistant animals. These sera were mixed with dilutions of relaxin preparation 123–1, the concentrations ranging from 0 to 5 mg. relaxin per cc. of serum. Precipitates appeared only in the tubes containing mixtures of relaxin and serum from resistant guinea pigs, the maximum precipitation occurring in the tube corresponding to 0.5 mg. relaxin per ml. of serum. Fresh samples of the two sera were then incubated with 0.5 mg. of relaxin per cc. of serum as described, centrifuged and the supernatants assayed for relaxin activity. Both sera showed relaxin titers that would be expected for the amount of relaxin added. It would thus appear that the mechanism of the acquired resistance to relaxin cannot be explained on the basis of a simple antibody formation.

DISCUSSION

The first attempt to measure the response of guinea pigs to relaxin in a semi-quantitative fashion was made by Fevold, Hisaw and Meyer (1930). They defined a unit as the minimum amount which, when injected into guinea pigs in full estrus, would cause definite loosening of the pelvic ligaments within 10–12 hours. In 1944, Abramowitz *et al.* developed a more quantitative assay procedure after an extensive study of the factors affecting the response. They introduced the use of castrated estrogen-primed guinea pigs, and recorded the percentage of animals found definitely to be relaxed. Apparently, neither group of workers encountered consistent losses in responsiveness upon repeated relaxin injections.

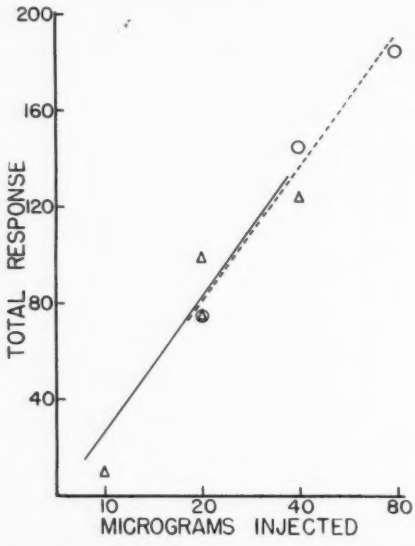

FIG. 2. Response to relaxin preparation MN-4 of two groups of guinea pigs when each was maximally sensitive. (— △— Group III; — ○— Group IV.)

The usual laboratory practice in performing relaxin assays has been to introduce new guinea pigs (after two priming courses of estrogen and relaxin) into the colony in a manner designed to assure their even distribution among all groups. Older animals were retired from the colony only when extensive tissue deposits around the symphyses made palpation difficult. Thus a test group of animals might be composed to individuals of various ages and assay histories, whose weights might vary between 300 and 800 gm. It is apparent from the results detailed above that the sudden addition of a relatively large number of fresh guinea pigs to an assay colony

may result in successive assays being performed with groups of substanstanially different sensitivities. Thus, considerable error might be introduced in the comparison of one preparation with another, or in replicate assays of a single preparation. It would appear that quantitative estimates of relaxin concentrations can reliably be made only with animals which are subjected to frequent standardization.[4] The latter should include determination of group sensitivity as well as assurance that a dose-response curve of satisfactory positive slope can be obtained.

Modified Assay Procedure and a Proposed Relaxin Standard.—A guinea pig unit of relaxin was originally defined as the quantity which, under the prescribed assay conditions, will produce "unquestionable symphyseal mobility" in $\frac{2}{3}$ of a group of 8–12 guinea pigs (Abramowitz *et al.*, 1944). Later, the assay was modified to include an assessment of the degree of response of each animal. Our results suggest that this definition should be further qualified to include either specification of previous assay history or the direct comparison in the same group of animals, with a standard relaxin preparation.

The peak sensitivity of different groups of guinea pigs appears to be remarkably uniform, as evidenced by the data of Figure 2. Here are recorded the results of assays of relaxin preparation MN-4 in two different groups of guinea pigs when each was maximally sensitive. The two curves are in excellent agreement, both as to slope and relative position. One guinea pig unit of relaxin, defined as the amount corresponding to a total response of 120, is represented closely in each instance by 30–35 micrograms of MN-4.

It is evident from the facts outlined above that it would be very desirable to have available a carefully prepared and carefully assayed relaxin standard. The availability of such a standard would greatly facilitate intra- and inter-laboratory comparisons of various relaxin preparations. Our experience indicates that such a standard should be assayed in the following manner: Forty to sixty immature virgin female guinea pigs are to be ovariectomized and then rested until reaching an average weight of about 350 gm. They should then be given a daily subcutaneous injection of 1 μg. of estradiol-17β dissolved in 0.05 cc. of sesame oil for four days. On the morning of the fifth day, all animals should be palpated and any obviously relaxed animals discarded. The others are divided into four to five groups (depending upon the number available) of not less than 10 animals each. Solutions of the standard are prepared by serial dilution, the concentrations being chosen so that the administered doses will fall on each side of the expected 1 unit level. A dilution is assigned to each group and the individual pigs are injected subcutaneously with 1 cc. of the solution. All

[4] R. L. Kroc (1953) has called attention to the necessity of checking the "eligibility" of the guinea pigs used for relaxin assay.

animals are to be palpated 6 hours later,[5] and the total response of each group calculated and plotted as previously described (Frieden and Hisaw, 1950). The assay procedure should be repeated on alternate weeks until the dose response curve reaches a maximum slope and indicates maximum sensitivity. The potency of the standard may then be calculated.

SUMMARY

A characteristic pattern of changing sensitivity has been exhibited by guinea pigs being used for relaxin assay. A rapid increase in sensitivity to relaxin during the first two to three test doses is followed by a period of relative constancy, after which the sensitivity gradually diminishes until a completely refractory condition is reached. Under the conditions of our experiments, the refractory state is reached within 3–6 months.

The decreased sensitivity to relaxin is not reversed by increasing the amount of estrogen used for priming. Although the development of a relaxin-specific antibody as a possible cause has not been ruled out, experiments designed to test this possibility gave negative results.

Some qualifications have been introduced into the accepted relaxin assaay procedure in order to circumvent the additional variable of diminished sensitivity. The need for a standard relaxin preparation is emphasized, and a method proposed for its standardization.

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[5] It is recommended that the solutions be coded and that the identity of each remain unknown to the operator until the assay is completed.

EVIDENCE OF SEPARATE HYPOTHALAMIC CENTERS CONTROLLING CORTICOTROPIN AND THYROTROPIN SECRETION BY THE PITUITARY[1]

MONTE A. GREER[2] AND HOWARD L. ERWIN

National Cancer Institute, Bethesda, Md.

PREVIOUS investigations have indicated that the hypothalamus controls the secretion of various pituitary hormones, including thyrotropin and corticotropin (Bogdanove and Halmi, 1953; Greer, 1952; Harris and Jacobsohn, 1952; Hume, 1952; McCann, 1953). Amphenone (1,2 bis-(p-aminophenyl)-2-methyl-propanone-1-dihydrochloride) has been shown by Hertz, *et al.* to cause marked adrenal and thyroid hypertrophy (Hertz, Allen and Tullner, 1950). They have also shown that adrenal hypertrophy can be selectively prevented by cortisone administration without impairing the goitrogenic effect and that thyroid hypertrophy can be prevented by thyroxin administration without impairing adrenal enlargement in the amphenone-treated animals (Hertz, Tullner and Allen, 1951). Both effects are lacking in the hypophysectomized animal (Hertz, Allen and Tullner, 1950). It is presumed that amphenone causes adrenal and thyroid hypertrophy by inhibiting the formation or secretion of their respective hormones, thus causing increased secretion of thyrotropin and corticotropin.

In the present study, amphenone was administered to rats with bilateral hypothalamic lesions to see if this would interfere with the usual response to the drug.

MATERIALS AND METHODS

Bilateral hypothalamic lesions were produced in adult female Holtzman rats with a modified Krieg machine (Greer, Mencken and Bolen, 1955) by a technique previously described (Greer, 1951). One to three weeks postoperatively the operated animals and a comparable control group were administered amphenone, 200 mg. per kg. body weight daily by stomach tube, for 10 to 14 days. At the end of that time the animals were killed with chloroform and adrenals and thyroids cleanly dissected and weighed on a Roller-Smith 200 mg. torsion balance. The glands were then fixed in formalin. Sections were made at 10 micra and stained with hematoxylin and eosin. The heads were removed and trimmed, most of the calvarium removed and the head then fixed in formalin for one

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[2] Present address: Radioisotope Research Section, Veterans Administration Hospital, Long Beach, California and Department of Medicine, University of California, Los Angeles.

week. Serial sections of hypothalamus and pituitary were made at 10 micra, each tenth section being mounted. The brain tissue was stained with toluidin blue for 6 hours, the pituitaries with hematoxylin and eosin. Eight separate experiments, utilizing a total of 51 control animals and 68 animals with hypothalamic lesions, were performed in this study. Body weights at the initiation of treatment with amphenone and at autopsy are reported.

Groups A, B, D and L had lesions just above the median eminence. Groups C, J, K, N and O had lesions in the median eminence. Groups E, F, G, H, I and M had lesions in the posterior hypothalamus or juncture of mid- and posterior hypothalamus. The lesions were placed as indicated in Table 1. Anterior coordinates are referred to the ear bars as O. Vertical and lateral coordinates are referred to the base of the skull and the median sagittal suture, respectively, as O. All lesions were made with a current of 6 ma. for 15 seconds with each insertion of the needle with the exception of Groups L and M in which 6 ma. for 30 seconds was used bilaterally.

TABLE 1. BODY AND ORGAN WEIGHTS OF RATS GIVEN AMPHENONE

Exp.	Group	No. rats	Body wt., gm.		Thyroid, mg.	Adrenals, mg.	Lesions		
			Initial	Final			Ant.	Vert.	Lat.
I	A	5	328.0± 5*	291.0± 6	18.1± 1.4	125.7±10.9	6.5	0.5	0.5
	Control	5	272.0± 6	226.4±15	43.4± 2.0	160.7±26.4			
II	B	6	262.0±13	204.0±13	16.8± 1.1	123.2±18.5	6.0	0.5	0.5
	C	5	282.0±16	204.0±36	21.9± 3.2	142.4±22.6	6.0	0	0.5
	Control	5	260.0±14	228.0± 5	41.5± 1.9	150.9±17.6			
III	D	6	295.0± 8	270.0± 9	17.6± 1.4	140.0±15.4	6.5	0.5	0.5
	Control	6	265.0± 4	223.0± 8	45.7± 2.8	152.1±18.2			
IV	E	2	221.0±21	238.0±12	41.5± 2.5	136.5± 7.2	4.0	0.5	0.5 & 1.0
	F	3	242.7±13	240.0±17	47.1±11.9	112.8±10.5	4.3	0.5	0.5 & 1.0
	G	6	270.0±11.6	260.8±22.2	30.8± 6.7	95.6± 8.4	5.5	0	0.5
	Control	6	256.3± 4	261.5± 5.6	39.3± 4.0	114.8± 6.4			
V	H	4	230.5±24.8	231.0±20.5	30.5± 5.6	96.7±14.7	4.7	0.5	0.5 & 1.2
	Control	5	253.6± 5.1	236.0± 9.6	42.1± 5.6	112.6± 9.6			
VI	I	5	281.6±15.3	213.4± 9.6	31.0± 2.1	105.9± 7.9	5.5	0	0.7
	J	2	326.0± 0.0	233.5± 1.5	17.3± 2.7	62.0± 6.0	6.0	0	0.7
	Control	6	252.7± 5.0	233.7±13.0	41.1± 1.5	111.3± 6.6			
VII	K	6	226.3± 6.3	232.5±10.0	18.1± 1.3	79.0± 4.3	6.0	0	0.5
	L	3	267.0±17.7	255.0±18.7	18.2± 2.3	99.7±25.2	6.0	0.5	0.5
	M	3	217.3±13.3	197.3±10.0	23.7± 1.8	104.4± 7.4	5.6	1.0	0.5
	Control	6	220.0± 3.3	220.0± 4.3	42.6± 2.8	108.4± 3.2			
VIII	N	7	224.1± 9.3	191.1± 9.6	13.8± 2.0	114.7±11.4	6.0	0	0.7
	O (untreated)	5	247.0± 8.2	259.2± 8.4	11.4± 1.1	41.1± 3.5	6.0	0	0.7
	Control	6	210.7± 4.0	213.0± 4.3	25.7± 2.7	128.4± 4.2			
	Control (untreated)	6	208.1± 3.1	217.5± 4.8	11.9± 0.4	62.8± 3.2			

* Standard error of mean.

RESULTS

All lesions in the median eminence or just above it markedly inhibited the goitrogenic response to amphenone exhibition (Table 1). Lesions in the posterior hypothalamus did not affect the goitrogenic response except for moderate inhibition when the lesions approached the region of the median eminence. In striking contrast to this was the failure of the lesions to inhibit significantly the adrenal hypertrophy caused by amphenone, with the exception of Groups J and K which had lesions in the median eminence. Groups C and N, however, also with extensive lesions in the median emi-

nence did not have significant impairment of the adrenal hypertrophy in response to amphenone. Histologic examination of the adrenals showed the usual hypertrophy and hyperplasia seen in amphenone-treated animals with the exception of Groups J and K. The thyroids of the hypothalamic animals, however, excluding those with posterior lesions, were indistinguishable from the glands of untreated control animals, in contrast to the marked hyperplasia exhibited in the amphenone-treated intact controls.

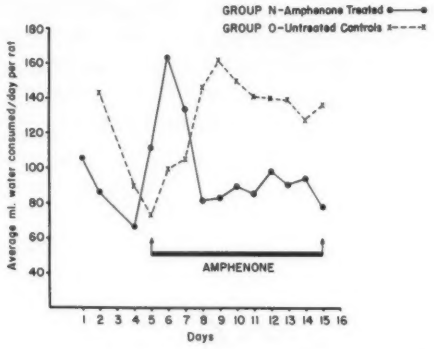

FIG. 1. Daily water consumption of rats with hypothalamic lesions (Experiment VIII).

Groups N and O, in addition, had measurements made of the daily water consumption (Fig. 1). Diabetes insipidus was produced in both groups. Only group N received amphenone. Shortly after the inception of treatment with this drug a reduction in the water intake was noted. This was probably related to the considerable weight loss which the animals underwent during amphenone treatment. The water intake was not measured in the other groups.

In Experiment 8, total adrenal fat and cholesterol analyses were made by Miss Lois Hallman. In both the intact and lesioned rats fed amphenone the customary marked increase in adrenal lipid was noted (Table 2). Although the total fat and cholesterol were somewhat higher in the control rats with hypothalamic lesions than in the untreated intact controls, the

TABLE 2. ADRENAL TOTAL LIPID AND CHOLESTEROL IN EXPERIMENT VIII

Group	Hypothalamic lesion	Amphenone	Adrenal wt., mg.	Total lipid %	Cholesterol %
Control	0	0	62.8± 3.2*	13.4±0.5	4.00±0.82
Control	0	+	128.4± 4.2	21.3±1.9	11.78±1.3
O	+	0	41.1± 3.5	16.3±1.2	6.47±0.65
N	+	+	114.7±11.4	26.8±0.9	14.1 ±0.73

* Standard Error.

absolute values were not significantly different. The difference may have been due to the smaller adrenal weight in the lesioned animals.

A marked reduction in the number of cells in the supraoptic and paraventricular nuclei was frequently seen in the animals with median eminence lesions. This seemed to bear little relation to whether the adrenals would become enlarged under amphenone treatment, however. Some animals with almost complete atrophy of supraoptic and paraventricular nuclei would have huge adrenals while in a few animals whose adrenals did not show any hypertrophy these nuclei showed little change from normal.

The pituitaries in general had no gross abnormalities in their morphology other than the hypertrophy of the intermediate lobe and atrophy of the posterior lobe noted by Bogdanove and Halmi (1953). In a few there was an area of infarction present in the lateral anterior lobes similar to that

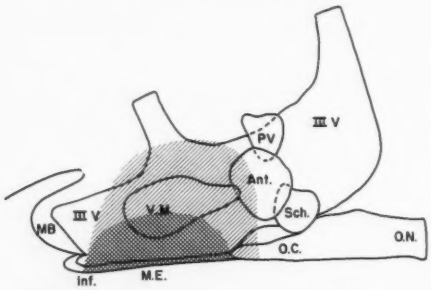

FIG. 2. Semidiagramatic representation of rat hypothalamus in a midsagittal projection. Hatching indicates area affecting thyrotropin secretion, cross-hatching indicates area destroyed when adrenal hypertrophy due to amphenone is inhibited. Ant. = anterior hypothalamic area; Inf. = infundibulum; M.B. = mammillary bodies; M.E. = median eminence; O.C. = optic chiasm; O.N. = optic nerve; P.V. = paraventricular nucleus; Sch. = suprachiasmatic nucleus; V.M. = ventromedian nucleus; III V = third ventricle.

previously noted (Greer, 1951). These infarcts were usually quite small, however, and did not seem correlated with the presence or absence of adrenal hypertrophy in response to amphenone.

DISCUSSION

These results confirm the earlier reports that anterior hypothalamic lesions will prevent thyroid hypertrophy resulting from administration of goitrogenic compounds (Bogdanove and Halmi, 1953; Greer, 1951). They also show that lesions placed in the area described do not interfere significantly with the adrenal hypertrophy caused by amphenone with the exception of an inconstant inhibition of adrenal hypertrophy caused by lesions in the median eminence.

Hertz *et al.* have shown that hypophysectomy or cortisone will prevent adrenal hypertrophy from amphenone administration. Amphenone will

not cause adrenal hypertrophy in hypophysectomized rats whose adrenals are maintained with corticotropin (Hertz and Tullner, unpublished data). It is thus quite likely that the hypertrophy is due to an increased secretion of corticotropin by the pituitary. It appears, therefore, that the hypothalamic lesions usually did not interfere appreciably with the secretion of corticotropin but that anterior lesions did markedly inhibit thyrotropin secretion. The hypothalamic areas controlling thyrotropin and corticotropin secretion by the pituitary would thus appear to be discrete. This confirms earlier evidence suggesting that independent hypothalamic areas control the secretion of various pituitary hormones (Bogdanove and Halmi, 1953; Greer, 1952; Greer, 1953). The area controlling thyrotropin is limited to a midline region between the paraventricular nuclei and median eminence (Greer and Erwin, 1954). Only lesions in the median eminence have so far been found to interfere with corticotropin secretion.

It is of interest that McCann (1953) has found that destruction of the median eminence would interfere with the eosinophil fall and adrenal ascorbic acid depletion in response to epinephrine and laparotomy in the rat but that the adrenals would not become atrophic. Similar results were reported by Hume (1954) in the dog. Ganong and Hume (1954) have also reported that lesions destroying all or part of the median eminence will prevent compensatory hypertrophy of the remaining adrenal following unilateral adrenalectomy in the dog. No adrenal atrophy due to these lesions was noted, however.

The results of the present study indicate that extensive destruction of the median eminence may not interfere with the production of a considerable degree of adrenal hypertrophy. It also appears from the data in Experiment 8 that, in spite of this, a significant degree of adrenal atrophy can be produced by means of similar lesions.

It has recently been suggested by McCann and Brobeck (1954) that pitressin is the substance controlling corticotropin secretion. The results with Groups N and O in this series do not seem to lend support to this hypothesis. In both groups there was relatively complete destruction of the anterior median eminence. In Group O there was considerable reduction in adrenal size compared with the untreated controls. In spite of this, however, the adrenals hypertrophied to approximately the same extent in the amphenone-treated animals with median eminence destruction in Group N as in the amphenone-treated normal animals. Both groups had rather marked diabetes insipidus. A reduction in water intake was induced in Group N by amphenone treatment and it is possible that this may have been due to increased release of pitressin by the hypothalamus. However, it is more likely that the reduction in water intake was due to the reduced food intake and weight loss caused by amphenone treatment. In any event, coupled with the lack of correlation between atrophy of supraoptic and paraventricular nuclei and absence of amphenone induced adrenal hyper-

trophy, the results cast some doubt on the role of pitressin in controlling corticotropin secretion.

SUMMARY

Bilateral anterior hypothalamic lesions prevented the goitrogenic response to amphenone administration in the rat but did not significantly interfere with the adrenal hypertrophy caused by amphenone. Destruction of the median eminence sometimes inhibited amphenone-induced adrenal hypertrophy and at other times did not. Posterior hypothalamic lesions did not significantly affect corticotropin secretion and inhibited thyrotropin secretion only when they encroached upon the median eminence. This indicates that the hypothalamic centers controlling thyrotropin and corticotropin production by the pituitary are discrete. The area controlling thyrotropin is located more anteriorly and above that controlling corticotropin. The corticotropin area seems limited to the region of the median eminence. It is suggested that pitressin may not play a significant role in the control of corticotropin secretion.

Acknowledgment

We are indebted to Dr. Roy Hertz for valuable advice and suggestions.

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NOTES AND COMMENTS

EFFECTS OF ASCORBIC ACID PRETREATMENT ON THE RESPONSE OF BLOOD GLUCOSE AND ADRENAL CHOLESTEROL IN THE INTACT RAT TO INSULIN

An influence of large amounts of ascorbic acid in the response of the adrenal gland and total organism to stress has been variously proposed and denied (Dugal and Thérien, 1949; Thérien *et al.*, 1949; Booker *et al.*, 1951; D'Angelo *et al.*, 1955; Knobil and Fregly, 1955). Presumably, ascorbic acid interacts with or modifies processes in tissues that promote adjustments of the organism, via mechanisms yet unknown, to noxious conditions. Experimental studies indicate that ascorbic acid is involved in carbohydrate metabolism since high blood sugar (Sigal and King, 1936), decreased glucose tolerance and low liver glycogen (Banergee and Ghosh, 1946) were reported in the scorbutic guinea pig. It may be noted in this regard that a decreased insulin sensitivity is one of the notable metabolic alterations found in stress (Engle, 1951). This paper reports the changes in blood glucose levels and adrenal cholesterol concentration in ascorbic acid pretreated rats following insulin administration.

METHODS

Male rats weighing 350–400 gm. were used. A laboratory chow and tap water were provided ad lib. prior to and during the experiment. Single, daily, subcutaneous injections of 50 mg. or 250 mg. of ascorbic acid[1] per 100 gm. body weight or an equivalent amount of saline were administered on four successive days. One hour after the regular injection on the 4th day a prolonged anesthesia was induced with nembutal and sodium barbital (Semonsen and Sawyer, 1954) to avoid effects of non-specific stimuli affecting the information obtained during the remainder of the experiment. Regular insulin (0.24 units per rat) or saline (0.2 cc.) was injected subcutaneously. Tail blood for glucose determinations (Nelson, 1944) were obtained directly before insulin injection and then at 30, 60, 90 and 120 minutes from rats pretreated with 50 mg. of ascorbic acid and their saline controls. Tail blood glucose determinations also were done in groups of rats pretreated with 50 mg. or 250 mg. of ascorbic acid and their saline counterpart immediately before insulin injection and at 1, 2, 3, and 4 hours. In addition, rats pretreated with both levels of ascorbic acid were given a single saline injection, s.c. and subjected to the same procedures as above mentioned. Equal numbers of rats representing each category of pretreatment were used concurrently. Immediately after the last tail blood sample was obtained, heart blood for ascorbic acid (Roe and Keuther, 1943), a specimen of liver for glycogen (Good, *et al.*, 1933), and the adrenal glands for cholesterol determinations (Schoenheimer and Sperry, 1934) were taken.

RESULTS

Blood ascorbic acid concentration in ascorbic acid-pretreated rats was 6–8 times greater than controls. Liver glycogen, adrenal weight, and adrenal cholesterol-total, free and esterified were not deviate in ascorbic acid and saline-pretreated rats at 2 and 4 hours after insulin injection or ascorbic acid pretreated rats not administered insulin (Table 1). The latter observations are in accord with the findings of Skelton and Fortier (1951).

The pattern of blood glucose changes after insulin were not different in ascorbic acid and saline-pretreated rats observed for 2 and 4 hours (Figs. 1 and 2). Glucose levels were maximally depressed (approx. 40% from pre-insulin levels) between 1 and 2 hours after

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[1] Sodium ascorbate (Abbott Laboratories), 500 mg. per cc.

TABLE 1. EFFECT OF ASCORBIC ACID PRETREATMENT ON LIVER GLYCOGEN, ADRENAL WEIGHT AND ADRENAL GLAND CHOLESTEROL CONCENTRATION 2 AND 4 HOURS AFTER INSULIN ADMINISTRATION

	Experiment I*		Experiment II a*			Experiment II b		
	Saline Pre-R_x	Ascorbic Pre-R_x	Saline Pre-R_x	Ascorbic	Pre-R_x	Saline Pre-R_x	Ascorbic	Pre-R_x
	Insulin	Insulin	Insulin	Insulin	Saline	Insulin	Insulin	Saline
Blood ascorbic acid, mg.%	—	2.1[1]	0.58	3.64§	4.14§	0.74	3.99§	4.25§
Liver glycogen gm.%	2.55	1.9	2.45	2.60	2.51	1.29	0.89	0.57
Adrenal wt., mg. per 100 gm. b.w.	8.66	9.25	9.83	10.60	9.25	9.07	9.60	10.30
Adrenal cholesterol mg./100 gm. adrenal								
Total chol.	35.95	31.38	34.95	34.76	29.39	27.80	30.59	29.00
Free chol.	2.00	3.42	3.34	3.47	3.18	1.53	2.06	2.69
Ester Chol.	34.04	27.96	31.61	31.28	26.17	26.30	28.50	26.30

* Experiment I: tissues obtained 2 hrs. after insulin administration; Experiment II: tissues obtained 4 hrs. after insulin or saline administration. See text for ascorbic acid pretreatment dose and injection schedule.
[1] All figures are means from 6 animals in each group.
§ All means were tested for statistical difference but only those indicated by this § sign were found to be significantly different from values of saline-pretreated groups.

insulin in saline and ascorbic acid-pretreated groups. It is unlikely that a difference in insulin sensitivity was extant, but masked, in the latter animals since the pre-insulin glucose levels were not significantly different in the groups with both types of pretreatment.

DISCUSSION

Allegretti and Vukadinović (1954) reported that four hourly injections of large amounts of ascorbic acid *after* insulin administration caused an increased insulin sensi-

Figures 1 and 2. Blood glucose response to insulin in rats pretreated with ascorbic acid. Values are Mean (±S.E.) per cent difference from pre-insulin glucose level of 6 animals in each group. *A:* Ascorbic acid pretreated following saline injection; *B:* Saline pretreated following 0.24 unit insulin; *C:* Ascorbic acid pretreated following 0.24 unit insulin.

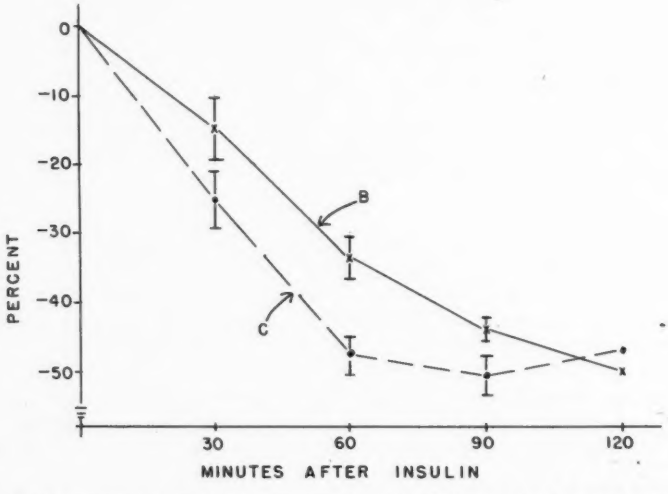

FIG. 1. Rats pretreated with 50 mg. ascorbic acid/100 gm. body weight for 4 days.

tivity in intact rats but not in adrenalectomized rats (Allegretti *et al.*, 1955). The difference in glucose responsiveness to insulin in the intact rat reported by the former authors and us is not readily explainable since the experiments are not exactly comparable in conception nor many details of execution.

The results of this study show that ascorbic acid pretreatment failed to modify insulin sensitivity or affect liver glycogen and post-prandial blood glucose concentrations. Moreover, there was no indication that exogenous ascorbic acid exerted an influence on gross adrenocortical activity, as reflected in adrenal gland cholesterol concentration, in response to insulin-induced hypoglycemia. It appears improbable, therefore, that a change in insulin responsiveness can be offered to explain the reported influence of administered ascorbic acid in the response of the organism to stress. It is suggested that the contradictory status of this subject might be resolved by the concept that studies of the effects of ascorbic acid on metabolic parameters associated with stress phenomena would elucidate the relationship of vitamin C and the response of the organism to stress, if it exists.

SUMMARY

Ascorbic acid pretreatment (at levels of 50 mg. or 250 mg. per 100 gm. body weight for 4 days) in intact rats failed to modify the response of the blood glucose to insulin

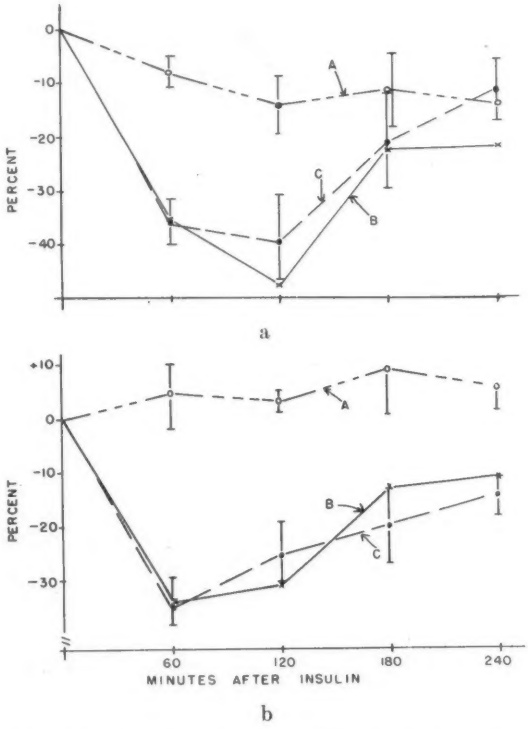

FIG. 2a: Ascorbic acid pretreatment as above; Fig. 2b: Rats pretreated with 250 mg. ascorbic acid/100 gm. body weight for 4 days.

during the course of 4 hours of observation. Likewise, other parameters of carbohydrate metabolism appeared unaffected by ascorbic acid pretreatment; and the response of adrenal cholesterol concentrations to insulin-induced hypoglycemia was not different from saline-pretreated rats.

MURIEL DURY AND ABRAHAM DURY

Dorn Laboratory for Medical Research
Bradford Hospital
Bradford, Pennsylvania

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PITUITARY-ADRENAL CONTROL OF SODIUM FLUX ACROSS FROG SKIN[1]

The well-known ability of the isolated skin of the frog to transport sodium ion against a gradient of electrochemical potential is influenced by extracts of the posterior lobe of the pituitary gland (Fuhrman and Ussing, 1951) and by adrenaline (Koefoed-Johnson, Ussing and Zerahn, 1952). The general activity of certain adrenal steroids on salt balance in mammals leads one to suspect that these substances might also act on sodium transport across frog skin, and that such effects might be of value in the study of the mechanism of adrenal steroid action in general. There are reports of effects of desoxycorticosterone glucoside on frog skin potentials (Taylor and Weinstein, 1952), and on water uptake by whole toads (Pasqualini and Risau, 1951), but Huf and Wills (1953) and Krueger in this laboratory (unpublished) were unable to show any direct effect of adrenal cortex extracts on isolated skins; Huf and Wills (1953) did find that pretreatment of frogs with adrenocorticotrophic hormone caused a decrease in salt uptake. Injections

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[1] This work was done in connection with contract Nonr 692(00) between the Biology Branch, Office of Naval Research, and the University of Oregon.

of cortex extracts into tadpoles of the bullfrog *Rana catesbeiana* cause a decrease in ash content which is partly attributable to decreased sodium content, but no changes in sodium excretion could be established (Neiland, unpublished).

Surgical removal of the interrenal glands has been reported by several investigators; Maes (1936), Cerf (1955) and Fowler and Jones (1955) report that in frogs an asthenia follows this operation, and Cerf (1955) attributes this to changes in the nervous system resulting from an altered sodium/potassium ratio in the blood. The operation of interrenalectomy involves unavoidable damage to the kidney circulation, however, and there is no certainty that accessory interrenal tissue has been removed. We therefore took advantage of the fact that the interrenals of frogs, like the adrenal cortex of higher verte-

TABLE 1. EFFECT OF HYPOPHYSECTOMY AND ACTH INJECTIONS ON POTENTIAL AND SODIUM FLUXES ACROSS ISOLATED SKIN OF *RANA PIPIENS*

Influx refers to movement from epidermis to corium; the corium is electrically positive to the epidermis. Values are means for the first two hours of measurement.

Treatment	Normal	Hypophysectomized	Normal, ACTH injected	Hypophysectomized ACTH injected
Potential difference, millivolts	31.8	33.0	32.8	44.7
Sodium Influx†	34.5	39.4	34.8	28.2
Sodium Outflux†	1.5	9.8	3.0	2.2
Net Transport†	33.0	29.6	31.8	26.0
Flux Ratio	23.0	5.0	11.6	12.8

Analysis of variance

	Source of Variance	Degrees of freedom	Sum of squares	Variance	Variance ratio
Potential Difference	Total	59	8,829.00		
	Treatments	3	1,265.55	421.85	3.12*
	Remainder	56	7,563.45	135.06	
Influx	Total	53	16,995.50		
	Treatments	3	2,923.63	974.54	3.46*
	Remainder	50	14,071.87	281.47	
Outflux	Total	29	1,102.17		
	Treatments	3	441.67	147.22	5.79**
	Remainder	26	660.50	25.40	

† Arbitrary units.
* Significant at the 5% level.
** Significant at the 1% level.

brates, are under pituitary control (Atwell 1935, 1937, Tuchman-Duplessis, Delgatte and Delgatte, 1955). We compared skin potentials, and sodium influx or outflux, in normal frogs (*Rana pipiens*), hypophysectomized frogs, normal frogs injected with adrenocorticotrophic hormone (ACTH) and hypophysectomized frogs similarly injected. Only the anterior pituitary was removed, and only results from those frogs which showed, on post mortem examination, complete absence of anterior pituitary tissue were used in the analysis of Table 1. It should be noted that the hypophysectomized animals showed no signs of asthenia, contrary to the observations of Fowler and Jones (1955) on *R. temporaria*. Two weeks after the operation, the skins were removed from the animals prepared and mounted as described by Fleming (1955) in the small-volume chamber designed by him. Influx and outflux were measured on separate skins with Na^{22}.

If we accept the 1% level of statistical significance, it appears from Table 1 that

hypophysectomy is followed by a distinct increase in the outflux of sodium ion across the skin. This may be interpreted as an increase in the passive permeability of the skin to sodium ion. Treatment of hypophysectomized animals with ACTH returns the outflux essentially to normal. We may therefore with reasonable confidence attribute the control of outflux to interrenal secretions. Treatment of normal animals with ACTH was without effect on any of the quantities measured. At the 5% level of significance it also appears that hypophysectomy is followed by increased sodium influx. This might simply be the result of increased permeability to sodium ion, but the net transport inwards seems also to be increased; this suggests a second effect, on the transport mechanism itself. Once again, treatment with ACTH restores the influx and net transport to, and in this case appears to shift both below, the normal level. One may also note a somewhat higher potential difference across the skins of hypophysectomized animals treated with ACTH, as compared with any of the other groups.

We would at present not wish to stress the last-mentioned effects, but conclude that the secretions of the interrenal glands of frogs normally hold the passive sodium permeability of the skin at a low level, and that hypophysectomy, by removing the source of the corticotrophic hormone, results in an increase in permeability. It should be noted that we found no significant correlation between either influx or outflux and skin potential, in contrast with the conclusions of Huf and Wills (1953). Further experiments to extend these observations are in progress.

MYERS, ROSE MARIE, WARREN R. FLEMING AND BRADLEY T. SCHEER
Department of Biology, University of Oregon
Eugene, Oregon

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AWARDS OF THE ENDOCRINE SOCIETY

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by the Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors.

Medal of The Endocrine Society

In 1954 the Council of the Society voted to establish a medal and an honorarium of $1,000 to be given to an individual for work of special distinction in endocrinology. The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada. In 1955 the Medal was presented to Dr. Carl R. Moore.

The Ciba Award

The Ciba Award to recognize the meritorious accomplishments of an investigator not more than 35 years of age in the field of clinical or preclinical endocrinology was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; 1945, Dr. Jane A. Russell; 1946, Dr. Martin M. Hoffman; 1947, Dr. Choh Hao Li; 1948, Dr. Carl Heller; 1949, Dr. George Sayers; 1950, Dr. Oscar M. Hechter; 1951, Dr. Albert Segaloff; 1952, Dr. Seymour Lieberman; 1953, Dr. Sidney Roberts and Dr. Claire Szego (Mrs. Roberts); 1954, Dr. Isodore M. Rosenberg; 1955, Dr. Jack Gross. Prior to 1952 the Award was $1,200. It is now $1,800. If within twenty-fourth months of the date of the Award, the recipient should choose to use it toward further study in a laboratory other than that in which he is at present working, it will be increased to $2,500.

The Ayerst, McKenna and Harrison Fellowship

This fellowship was established in 1947. It is designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. The fellowship is awarded on alternate years (1955, 1957, etc.) and the stipend which will not exceed $5,000 may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree or candidates for either of these degrees are eligible for appointment.

Applicants must submit the following information:

(1) Evidence of scientific ability as attested by studies completed or in progress.

(2) Recommendations from individuals familiar with the candidate and his work.
(3) A proposed program of study.
(4) Acceptance of the individual by the head of the department in which the Fellowship will be held.
(5) A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

The Schering and The Upjohn Scholars of The Endocrine Society

The Council of The Endocrine Society established a category of Scholars. These grants have been made available through the generosity of The Schering Corporation and The Upjohn Company, and will be awarded to established investigators and teachers in the fields of endocrinology who wish to extend their opportunities for work either in this country or abroad.

The award will not exceed $2,500 annually for each individual, and shall be granted on the basis of proposals submitted by the applicant and such applications should include the estimated financial needs. The funds may be used for travel, maintenance and other expenses.

Nominations

Nominations for the Medal of The Endocrine Society; the Ciba Award; and the Ayerst, McKenna and Harrison Fellowship may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 N. Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than November 1st each year.

Proposals for appointment as Scholars of The Endocrine Society should be made in writing by the individual, and addressed to the Secretary of the Society. They should be submitted by November 1, 1956.

The Awards Committee will meet in November and notice of awards to successful nominees and applicants will be made not later than December 1, 1956.

THE EIGHTH POSTGRADUATE ASSEMBLY IN ENDOCRINOLOGY AND METABOLISM

Sponsored jointly by the Endocrine Society; The University of Texas Postgraduate School of Medicine, and the University of Texas M. D. Anderson Hospital and Tumor Institute,
Houston, Texas.
October 2–27, 1956

The faculty will consist of twenty-two eminent clinicians and investigators from various parts of the country in the fields of endocrinology and metabolism. The program will cover the various endocrinopathies, with emphasis on the clinical aspects, demonstration of laboratory tests, presentations of cases, and question-and-answer panel discussions. The course is designed to cover the main aspects of diagnosis and therapy in the field of endocrinology and metabolism for the physician in general practice and for those in other specialties who wish to have a general knowledge of this rapidly growing field.

A syllabus with brief abstracts of lectures will be available to the registrants at the time of the assembly.

For further information concerning the program and registration, write to Dr. J. B. Trunnel, M. D. Anderson Hospital, 6723 Bertner Avenue, Houston 25, Texas. Registration is limited to 100; tuition fee is $100.00. Rooms will be reserved for the students and faculty at the Shamrock Hilton Hotel which is adjacent to the Texas Medical Center.

NATIONAL INSTITUTES OF HEALTH

NOTICE

The Public Health Service has announced a new procedure to expedite the processing of research grant applications for those requests which do not exceed $2000 plus indirect costs and which do not ask support for more than one year. Such applications will be accepted and processed on receipt and are not therefore subject to the usual deadlines for submission prior to review.

Council recommendations can be expected on these applications within 1–4 months from the time of submission. These procedures do not apply for requests for supplements to existing grants.

Address all applications as well as requests for forms or additional information to the Division of Research Grants, National Institutes of Health, Bethesda 14, Maryland.